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THE UNIVERSITY OF ALBERTA

CONTROL OF GAP JUNCTION FORMATION AND FUNCTION IN CANINE TRACHEAL SMOOTH MUSCLE

BY

© MATHUR S. KANNAN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Control of Gap Junction Formation and Function in Canine Tracheal Smooth Muscle, submitted by Mathur S. Kannan in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Date 8th February, 1978.

To

Lakshmi and my parents

ABSTRACT

Canine tracheal smooth muscle has been described as a multi-unit muscle (Bozler, 1948; and Kroeger and Stephens, 1975). Treatment of this muscle with Tetraethylammonium (TEA), a K^+ conductance blocker, results in a conversion from multiunit to single-unit type of behaviour. This study was designed to investigate the morphological bases for this conversion.

The results obtained demonstrate a functional cholinergic excitatory and a β-adrenergic inhibitory innervation to canine trachealis. Two types of varicosities were characterized on the basis of the distribution of vesicles. Nerve fibres or their varicosities were found in the space between bundles of smooth muscle cells.

The smooth muscle cells, as seen in thin sections under the electron microscope, were irregular in shape with many processes. Gap junctions were seen exclusively between processes of two cells and had a typical 7-layered appearance with a central 2 nm wide gap.

Treatment of tissues with TEA resulted in phasic mechanical activity and was accompanied by a significant increase in the number of gap junctions. The compound 4-Aminopyridine (4-AP), another K^+ conductance blocker, also induced phasic response and an increase in the number of gap junctions in canine trachealis. There was a correlation in time of the structural and functional changes brought about by TEA and 4-AP, but not with respect to the doses employed.

Responses to TEA were not affected by atropine. On the other

hand, the mechanical effects of 4-AP could be blocked by atropine. Atropine did not prevent the increase in the number of gap junctions brought about by 4-AP. Treatment of tissues with acetylcholine (Ach) did not result in either phasic mechanical activity or increased junction formation. The mechanical and structural effects of 4-AP appear thus to involve, in addition to releasing Ach, a direct smooth muscle action.

The rapidity of TEA- and 4-AP-induced junction formation suggested a mechanism independent of new protein synthesis. Inhibition of protein synthesis (to the extent of 95%) by cycloheximide (CHX) did not prevent increased junction formation in tissues where phasic mechanical activity to TEA and 4-AP was present. However, in tissues where such mechanical activity was not induced after similar treatment, no such increase in gap junctions was observed. These results were interpreted in the light of the extent of structural damage to cells observed in the CHX-treated tissues.

Continued formation of gap junctions in the absence of new protein synthesis in canine trachealis suggested a mechanism involving assembly of preformed subunits in the membrane. Freeze-fracture studies revealed gap junctions on processes of cells. Gap junctions were very rarely encountered in the replicas and the stages leading to their formation after TEA and 4-AP treatment could not be observed.

Gap junctions have been suggested to play a role in cell-to-cell electrical coupling in smooth muscle and their presence in canine trachealis may provide one necessary basis for such coupling. Their increase after treatment with TEA and 4-AP may provide better coupling between the cells. However, any such increased coupling was not by itself sufficient to induce single-unit behaviour. An attempt has been made to relate the structural effects to the electrophysiological changes seen after TEA-treatment. Study of the electrophysiological changes accompanying the action of 4-AP in the presence and absence of atropine would aid in elucidating the necessary and sufficient conditions for single-unit behaviour.

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CHAPTER I

GENERAL INTRODUCTION

CHAPTER I

GENERAL INTRODUCTION

A. REVIEW OF LITERATURE

Classification of smooth muscle: The vertebrate smooth muscles were divided into two categories by Bozler (1948).

i) Single-unit, which are usually spontaneously active, behave as a single synchronized unit in contractile function. Action potentials initiated in any cell are conducted to other cells. The smooth muscles of the gut, uterus and ureter were considered by Bozler to belong to this category.

ii) Multiunit smooth muscles, which are usually not spontaneously active, consist of independently acting units. These are dependent on activity of nerves and/or diffusion of mediator to each cell for initiation of excitation. Bozler included most vascular smooth muscles, nictitating membrane, iris sphincter, urinary bladder, tracheobronchial smooth muscles in this category.

Many of the multiunit smooth muscles were, in later studies, shown to exhibit single-unit characteristics, such as conducted action potentials. Guinea-pig vas deferens and bladder and many vascular smooth muscles are examples of this type (Burnstock, 1970).

A classification of smooth muscles based on the nature of autonomic innervation to the cells was proposed by Burnstock (1970). The smooth muscles were categorized into three types:

i) The smooth muscles in this group were considered to have innervation to all the cells with close neuromuscular contacts. This model would correspond to Bozler's multiunit category and is represented by vas deferens from mouse and rat and possibly cat ciliary muscle. The cells were thought to be connected through "low-resistance" contacts, through which there could be electronic spread of junction potentials and spike activity. The membrane properties would limit the spread of all-or-none action potentials in these tissues.

ii) Two types of cells were envisioned in this group. One type the directly innervated or "key" cells which are influenced by transmitter released from nerve endings closely situated to them. The other type is indirectly innervated or "coupled" cells, in which junction potentials are evoked by electrotonic spread from key cells. When excitation by nerves affects a large-enough area of the cell membranes of both cell types, a propagated action potential can occur. The smooth muscles like guinea-pig vas deferens, bladder, circular intestinal muscle, etc. were considered to belong to this category.

iii) The smooth muscles in this group behave like the unitary types proposed by Bozler. There are very few or no close junctions between the nerves and the cells in smooth muscles of this type. Three types of cells were described: a few directly innervated or "key" cells; a few "coupled" cells which are activated by electrotonic spread of junction potentials; and few "indirectly-coupled" cells activated by propagated action potentials from other cells. The longitudinal muscle of the gut, ureter, most vascular smooth muscles and possibly the uterus come under this classification.

The classification of Burnstock, for the most part, was purely speculative. The mechanisms of excitation of the smooth muscles in the three categories cannot be deduced from the description of the organization of the cells. There is also an implicit assumption that "low-resistance" contacts exist between the cells in all smooth muscles. The classification also seems to be rigid, much like the earlier one proposed by Bozler.

Innervation of smooth muscles:

Quantitative examination of neuromuscular contacts in different smooth muscles has been undertaken. In the vas deferens of adult rat (Richardson, 1962 and Taxi, 1965), mouse (Lane and Rhodin, 1964 and Yamauchi and Burnstock, 1969), and guinea-pig (Merrillees et al, 1963), the results of such studies suggest that individual cells are innervated. Close neuromuscular contacts have been described in these tissues (separation of about 200 A° between nerves and muscle cells). Smooth muscle like the circular coat of intestine was shown to have dense innervation, but not every cell seems to be innervated (Burnstock, 1970). Most vascular smooth muscles, uterus, ureter and the longitudinal layer of the intestine were shown to have sparse innervation. Finally, smooth muscles with no nerves have been described, like the muscle cells of the chick amnion (Burnstock, 1970). Thus, the density of innervation differs in different smooth muscle systems examined.

Tracheo-bronchial smooth muscles:

The smooth muscles of the respiratory tract from different species have been studied for the pattern and types of innervation. The excitatory innervation to the respiratory smooth muscle has been shown to be cholinergic (Mizers, 1955 and Widdicombe, 1963), from the vagal parasympathetic component of the autonomic nervous system. There is also an inhibitory innervation from the sympathetic system (Rikimaru and Sudoh, 1971). In addition, a third component of nervous activity, which has been described for the smooth muscles of the gastro-intestinal tract (Burnstock, 1973), has been shown in the respiratory smooth muscles of guinea-pig (Coburn and Tomita, 1973) and human (Richardson and Beland, 1972), the non-adrenergic and non-cholinergic inhibitory system (NAI). There is no evidence for the presence of this inhibitory innervation to the canine tracheal smooth muscle (Suzuki et al, 1976). Thus, the airway smooth muscles are thought to be regulated by both the parasympathetic and the sympathetic components of the autonomic nervous system acting in concert. With respect to the presence of non-adrenergic inhibition, there seem to be species differences.

The nervous regulation of the smooth muscles of the gastrointestinal as well as those of the respiratory tracts seem to be similar. The smooth muscle, epithelium, vagal innervation and the ganglion cells in the lung have been shown to have a common embryological origin with these systems in the gut (Krahl, 1964). Thus, it is not surprising that the neural control of both these systems are identical in some respects.

Electrophysiological, histochemical and ultrastructural studies on respiratory smooth muscle have been carried out to correlate the physiological responses to the innervation present. In the human tracheobronchial muscle, Richardson and Beland (1976) demonstrated an atropine-sensitive excitatory response and a Tetrodotoxin (TTX)-sensitive inhibitory response to field stimulation using appropriate parameters. There was no evidence for an adrenergic inhibitory influence with this technique, nor could the nerves be demonstrated by histochemical techniques. In the respiratory smooth muscle of the guinea-pig there was pharmacological evidence for the presence of adrenergic inhibition and the nerves could be demonstrated by histochemical techniques (Richardson and Bouchard, 1975).

In a correlated histochemical ultrastructural study on the cat tracheobronchial smooth muscle, Silva and Ross (1974) showed an extensive autonomic innervation. Fluorescence studies revealed a network of adrenergic fibres on the surfaces of the trachealis which also extended between the muscle cells. In cats treated with 6-Hydroxy dopamine (6-LHDA), an agent which has been shown to cause selective degeneration of adrenergic fibres (Thoenen _et al_, 1970), the fluorescence associated with adrenergic nerves was no longer seen. In trachealis muscle, nerve fibres with structurally different vesicles were noted: agranular vesicles (300 - 2000 A^o diameter); small (300 - 600 A^o) and large granular vesicles (700 - 1200 A^o). After 6-OHDA treatment, the nerve fibres contained predominantly agranular vesicles and very few large granular vesicles. Those nerve profiles which contained large granular and small granular vesicles were considered to be adrenergic nerves, since they were apparently destroyed by 6-OHDA treatment. The profiles remaining after 6-OHDA treatment, predominantly agranular vesicles and a few large granular vesicles, were considered to be cholinergic.

Cameron and Kirkpatrick (1977) produced evidence for a cholinergic excitatory innervation for the bovine tracheal smooth muscle. Using the single-sucrose gap apparatus to record potential changes, they recorded excitatory junction potentials (ejps) in response to electrical field stimulation. The ejps were followed by brief twitch-type contractions of the tissue strips. On repetitive stimulation, both the amplitude of the depolarizations and the tetanus were increased, with no evidence of facilitation or production of action potentials. External administration of acetylcholine (10^{-6} gm/ml) simulated the responses (both mechanical and electrical) to repetitive electrical stimulation. Neostigmine potentiated and atropine either reduced or abolished the contraction as well as the ejps. Thus, it was evident that the ejp and contraction were due to release of acetylcholine from the intrinsic nerve endings. Their electron microscopic studies revealed that the nerves were present in the clefts between smooth muscle cell bundles. The axonal varicosities were not seen in close proximity to the cells. Determination of the quantitative proportion of muscle cells and nerves revelaed a sparse innervation.

In the canine tracheal smooth muscle, Suzuki et al (1976) showed that the nerves bewteen the muscle cells were rare. The nerve fibres contained three kinds of vesicles: a) agranular vesicles (diameter 500 - 700 A°); b) small granular vesicles (diameter 600 - 800 A°); and c) large granular vesicles (diameter 1000 - 1500 A°). The nerve fibres with the agranular vesicles were reportedly more frequent than those containing granular vesicles. Their histochemical studies for acetylcholinesterase revealed a diffuse staining; also the catecholamine-fluorescence was localized mainly around the blood vessels and close to cartilage with few fluorescent fibres between the muscle cells.

The smooth muscle of the guinea-pig sphincter pupillae has been shown to have a very dense innervation (Gabella, 1974). In a full cross-section of the spincter, Gabella reported about 2791 profiles of muscle cells and 843 nerve fibres, more than half of which contained synaptic vesicles. About 194 out of the 843 nerve fibres were seen separated from muscle cells by a gap of about 20 nm. Post-junctional specializations were also noted along some of the closely located nerve endings. Based on the approximate length (7 μm) of a varicosity, Gabella reported that there could be more than half a million nerve terminals in one pupillary muscle. It was concluded that all muscle cells were innervated and many of them could be in close proximity to several varicosities.

In the vasa deferentia of rat and guinea-pig, Goto et al (1977) observed spontaneous junction potentials (sjps). They also reported a denser innervation and higher frequency of discharge of sjps in the rat vas deferens than in the guinea-pig muscle. They attributed the sjp frequency to the density of innervation of the muscle.

From the foregoing discussion it is evident that different visceral smooth muscles vary in their pattern and density of innervation. Smooth muscles like the pupillary muscles and the vas deferens, which are involved in rapid movements, are characterized by innervation of what appears to be every muscle fibre. On the other hand, in smooth muscles showing slow graded responses like those of the gut and the uterus, the innervation allows for diffuse release of transmitter from the nerve endings (Burnstock, 1970).

In smooth muscles where excitation is supposedly initiated by nerves and/or diffusion of transmitter from the terminals the density of innervation has been shown to be high (Bennett and Merrillees, 1966 and Gabella 1976). In smooth muscles where nervous influence is considered to regulate the spontaneous activity of the cells, the innervation has been found to be less dense (Bennett and Rogers, 1967). As cited above, for the bovine and canine tracheal smooth muscles, where initiation of excitation is considered dependent on nerves (in the absence of myogenic electrical activity), the density of innervation has been found sparse. Thus, the basis of excitation of smooth muscles cannot always be inferred from knowledge of the density of innervation.

Cameron and Kirkpatrick (1977) hypothesized the following scheme of events to be operative during neuro-muscular transmission in the tracheal smooth muscle: depolarization of more peripheral cells in the tissue by the acetylcholine released from parasympathetic postganglionic nerve fibres and electrotonic spread of the potential changes to cells in deeper bundles through low-resistance interconnections. The types of cell-to-cell contacts found in these muscles are discussed in a later section. Since the space constant of these tissues is long (see later section), it is conceivable that there could be nondecremental conduction of potentials.

General ultrastructure of smooth muscles:

Size and shape of smooth muscle cells:

The cells in different smooth muscles vary in size, but in any one tissue seem to be constant (Gabella, 1973). The diameter of cells in different smooth muscles has been reported (Burnstock, 1970) and varies from about 1.5 μm up to 6 μm. The cells in smooth muscles fixed in the relaxed and contracted states have different cross-sectional areas (Gabella, 1973 and 1976). During isotonic contraction, the smooth muscle cells of the guinea-pig taenia coli were shown to increase in the cross-sectional area. The percentage increase was found to be the same as the percentage decrease in length. In longitudinal sections of stretched tissues, the cells were shown to run parallel with smooth surfaces while in isotonically contracted tissues they were larger with numerous finger-like projections.

The smooth muscle cells of the gut are usually round or oval in transverse sections when fixed in the relaxed state. In some muscles, the cells have more irregular shapes. In the rat anococcygeus muscle, the surface membrane of the cells was shown to have large invaginations throughout the length of the muscle (Gabella, 1973). The cells of the guinea-pig sphincter pupillae also had very irregular shapes (Gabella, 1974). The surface to volume ratio, due to the shape, was shown to be very high. Trachealis smooth muscle cells were shown to have elliptical shapes in cross-section (Suzuki et al, 1976). These processes should be different from those observed by Gabella (1976) in smooth muscles fixed in the contracted state.

Surface membrane:

The surface membranes of smooth muscle cells have structures described as caveolae. Some caveolae open to the extracellular compartment, as demonstrated in studies using extracellular tracers (Gabella, 1973; Devine et al, 1972; and Garfield, 1973). In thin sections, some caveolae appear to be intracellular although this could arise due to the connection not being included in the plane of the section. In the longitudinal muscle of the mouse intestine, Rhodin (1962) reported that the caveolae increased the surface of the cells by 25% whereas in the guinea-pig taenia coli Goodford (1970) reported a 70% increase in surface.

A role for these caveolae in control of cell volume has been proposed by some investigators (Daniel and Robinson, 1970). The study by Garfield and Daniel (1977) reveals that the membrane vesicles in rat myometrium have properties in common with the volume pump described by Daniel and Robinson (1971) and Rangachari et al (1972) in this tissue. The maintenance of vesicles seems to be dependent on ATP formation and metabolism of the cells.

The caveolae have an average diameter of about 1000 A° (Gabella, 1973 and Garfield, 1973). They have been shown to appear very early in embryonic development (Gabella, 1973). It is thought that the vesicles delimit a space around the cells whose ionic composition is directly controlled by the cells themselves.

Cytoplasmic organelles:

The superficial regions of the cytoplasm, subjacent to the surface membrane and the caveolae, of many smooth muscle cells have abundant sacs and tubules of smooth sarcoplasmic reticulum. These have been observed in vascular smooth muscle (Somlyo et al, 1971) as well as in taenia coli, uterus, vas deferens, intestine and stomach smooth muscles (Gabella, 1971). In the taenia coli, Devine et al (1972) have estimated that the sarcoplasmic reticulum contributes about 2% of the total cytoplasmic volume. The presence of sarcoplasmic reticulum in smooth muscle just below the surface membrane and caveolae has prompted investigators to speculate a role in storage, release and transport of Ca^{++} in smooth muscle (Gabella, 1971 and Somlyo and Somlyo, 1971).

The deeper regions of the cytoplasm, especially at the poles of the nucleus, in smooth muscle cells are the smooth and rough sarcoplasmic reticulum and mitochondria. Mitochondria are also seen underneath the surface membrane.

Cell-to-cell contacts in smooth muscle:

The cells in different tissues come into close contact at certain regions and the structures of these various contacts have been the basis of some reviews (Weinstein and McNutt, 1972; McNutt and Weinstein, 1973; Friend and Gilula, 1972; Pappas, 1973; Furshpan and Potter, 1968; and Henderson, 1975). In smooth muscles, four major types of cell-to-cell contacts have been described: nexuses, intermediate contacts, interdigitations and simple appositions. The structure and functions of the nexus are discussed in a later section of this Chapter.

Intermediate contacts: These have been demonstrated after glutaraldehyde primary fixation followed by osmication and staining in smooth muscles. The plasma membranes of neighbouring cells are parallel with a separation of about 50 nm (range 40 - 70 nm) at these junctions (Henderson et al, 1971). The cytoplasm of the cells, just underneath the membranes at the junction, appears more dense. The extracellular space within the contact usually has a dense line and is granular in appearance. The intermediate junctions and desmosomes seen in epithelial cells (Farquhar and Palade, 1963) resemble the intermediate contacts in smooth muscle, although the first two types have narrower intercellular spaces.

Interdigitations: These appear as cylindrical or mushroom-shaped invaginations of one cell into a neighbouring cell, the shape being dependent on the plane of section. Gabella (1972) described them as "bulbous projections". The membranes of adjacent cells lose their basement membranes at these contacts and are separated by a gap of about 10 nm. There are no membrane specializations observed at these contacts. Interdigitations have been observed more commonly in tissues fixed with permanganate (Dewey and Barr, 1964; Oosaki and Ishii, 1964; Bergman, 1968; Nishihara, 1970; and Henderson et al, 1971). They also seem to be more frequent in contracted tissues (Rhodin, 1962; Caesar et al, 1957; Lane, 1965; and Nagasawa and Suzuki, 1967).

Simple appositions: The membranes of neighbouring cells run parallel, separated by a gap of about of 10 nm at these junctions (Henderson et al, 1971 and Gabella, 1972). Such contacts have been described in a variety of smooth muscles and they seem to be typical for small arterioles (see Henderson, 1975).

The precise physiological role for these three types of cell-to-cell contacts is not known. The possibility, however, exists that they could be potential sites of cell-to-cell coupling. There are no special characteristics of these junctions which make it possible to identify them in freeze-cleaved replicas.

Nexuses: The cell membranes of adjacent cells are sometimes seen in close apposition without a visible gap and with no electron-density of the subjacent cytoplasm at these junctions. Dewey and Barr (1962) called these nexuses or tight junctions. They claimed that the smooth muscle cell membrane outer leaflets fused at the nexus, giving a 5-layered appearance in thin sections of tissues fixed in permanganate and examined under the electron microscope. Colloidal lanthanum, an extracellular space marker, could penetrate some of the structures previously classified as nexuses suggesting that the outer membrane leaflets were separated by a gap, as in gap junctions between epithelial cells (Revel et al, 1967). The presence of a gap of about 2 nm was confirmed at the nexus of smooth muscle and hence the term gap junction was used to describe these contacts (see Gabella, 1973).

The presence and the extent of the gap between the apposing membranes have been attributed to the fixation and dehydration procedures used. There is no evidence to indicate more than one type of gap junction in tissues (Gabella, 1973). In tissues fixed with permanganate swelling of cells and fusion of membranes has been shown to occur, causing formation of nexus-like structures (Daniel et al, 1976). Brightman and Reese (1969) showed that permanganate fixation caused conversion of gap junctions into tight junctions in the central nervous system. Likewise, in mouse liver fixed in glutaraldehyde and dehydrated with acetone the gap of 2 nm was shown to disappear and the junction had a 5-layered appearance (Goodenough and Revel, 1970).

Fine structure of gap junctions:

a) Thin-section appearance: In thin section electron microscopy, the gap junction appears as a 7-layered structure with a central gap about 2 nm wide either after uranyl acetate en bloc staining (Revel and Karnovsky, 1967) or after filling the extracellular space with electron-dense tracer like lanthanum (Revel and Karnovsky, 1967). However, in lanthanum-impregnated tissues, a central zone about 55 A^o in width seems to rendered opaque with lanthanum in thin sections passing perpendicular to the plane of the gap junction due to staining of the membrane outer leaflets. In thin sections of lanthanum-impregnated cardiac muscle, when viewed en face, the nexus is seen to consist of subunits about 70 to 80 A^o wide. There is a central dot 15 A^o in diameter in these subunits (see McNutt and Weinstein, 1973). A similar lanthanum image of gap junctions has been confirmed by others (Brightman and Reese, 1969). The subunits of the gap junctions themselves are arranged in a hexagonal array with a cintre-to-centre spacing of 90 to 100 A^o (see McNutt and Weinstein, 1973).

b) Negative staining: In negatively stained isolated liver-cell plasma membrane fragments, Benedetti and Emmelot (1965) showed a hexagonal array of subunits. When Revel and Karnovsky (1967) described the structure of lanthanum-impregnated gap junctions, it was evident that the hexagonal array of subunits seen in liver plasma membrane was in fact the gap junction since it only occupied a small fraction of membrane fragments scanned (Benedetti and Emmelot, 1968). The hexagonal array of subunits was also demonstrated in hegatively stained isolated gap junctions from liver (Goodenough and Revel, 1970; and Goodenough and Stoeckenius, 1971).

c) Freeze-fracture appearance of gap junctions: Freeze-fracture studies of gap junctions reveal closely packed particles arranged in a hexagonal array with a centre-to-centre spacing of 9 to 10 nm when viewed on the membrane PF face. The membrane EF face has a hexagonal array of depressions or pits, which also have a centre-to-centre spacing of 9 to 10 nm (Chalcroft and Bullivant, 1970; Goodenough and Revel, 1970; and McNutt and Weinstein, 1970). However, the relationship between the subunits delineated by lanthanum in thin sections as well as by negative staining and the subunits seen in freeze-cleaved nexuses is poorly understood.

The particles seen on the PF face of nexuses are 6 to 7 nm in diameter, whereas the subunits seen in lanthanum-impregnated nexuses measure 7 to 7.5 nm. The measured diameters are thus similar. However, the particles seen in the replicas are actually enlarged in size due to capping with Platinum (Pt) during shadowing and in calculating the true dimensions a correction factor must be introduced.

The corrected size of the particles would be 4 to 5 nm in diameter (assuming that Pt deposition would increase the size by 1.5 to 2.5 nm depending on replica thickness). Thus, the particles seen in replicas are clearly smaller than the subunits delineated by lanthanum, although they are assumed to represent the same structure (McNutt and Weinstein, 1970).

Gap junctions and cell-to-cell coupling:

Electrotonic coupling between cells has been taken as evidence for the presence of gap junctions in many systems. Likewise, the presence of gap junctions between cells is considered as evidence for electrotonic coupling in tissues. In some systems, a temporal correlation between electrical coupling, as demonstrated by electrophysiological techniques, and the presence of gap junctions, as revealed by ultrastructure, has obtained.

Johnson et al (1974) studied the formation of gap junctions in reaggregating Novikoff hepatome cells in culture. Electrophysiological measurement of coupling in these cells was done concurrently. They examined the freeze-fracture replicas of the cells at different times after reaggregation and noted that establishment of coupling coincided with the appearance of particle aggregates in the membrane. The coupling coefficient was found to increase with an increase in the size of the particle aggregates.

Pappas et al (1971) and Asada and Bennet (1971), in their studies on crayfish giant axons, observed a temporal correlation of loss of coupling and disappearance of gap junctions when solutions containing low chloride were used. However, the changes were reversible; coupling was restored at a time when gap junctions reappeared.

In chick myogenic cells, presence of gap junctions and establishment of electrotonic coupling were seen to be temporally related (Rash and Fambrough, 1973 and Rash and Staehelin, 1974). Revel et al (1971) found other types of cell-to-cell contacts in the myoblasts and argued that their role in coupling should also be considered.

Human fibroblasts which showed coupling, gap junctions and contact inhibition of growth were hybridized with malignant mouse L cells which were not coupled, had no gap junctions and no contact inhibition (Azarnia et al, 1974). On culturing the hybrids, some cells were seen to lose the human chromosomes and they exhibited characteristics of the mouse L cells. These heterokaryons lost the capacity to form gap junctions and were not coupled. The hybrids, however, had gap junctions and were coupled. This also suggests that the ability to form gap junctions in these cells is genetically determined.

In a study by Gilula et al (1972), Chinese hamster fibroblasts which were incapable of incorporating purine nucleotides were cultured with cells which were competent in this respect. When the incompetent cells were coupled with the competent cells, they showed gap junctions and incorporated the purines. However, when ionic coupling was not established in some cultures, there were no gap junctions and the cells remained incompetent.

In cardiac muscle immersed in hypertonic solution, Barr et al

(1965) reported a block of spike propagation due to the disruption of nexuses. These results were contradicted by Dreifuss et al (1977) who attributed the block of spike activity to swelling of the sarcoplasmic reticulum.

In smooth muscle incubated in solutions made hypertonic by the addition of sucrose, Barr et al (1968) reported disruption of nexuses. They used permanganate fixation to demonstrate the nuxuses. Hypertonicity was claimed to result in loss of spontaneous spike activity as a result of loss of nexuses. Their results could not be confirmed by other investigators (Cobb and Bennett, 1969; Nishihara, 1970; and Daniel et al, 1976). Tomita (1967) showed that loss of spontaneous spiking was related to the hyperpolarizing effect of hypertonic solutions in the same tissue.

Electrical coupling had been shown between cells of many smooth muscles where gap junctions were also seen. On the other hand, some smooth muscles, which are electrically coupled, were shown not to possess gap junctions (Henderson et al, 1971; Daniel et al, 1976; and Gabella, 1973 and 1975). Thus, there is no convincing correlation between presence of coupling as well as gap junctions in all smooth muscles. So far no instances have been found in which gap junctions were present but coupling was absent. Thus, they may be a sufficient but not exclusive mechanism for cell-to-cell coupling in smooth muscles. In the circular layer of the dog small intestine, Daniel et al (1976) found gap junctions as demonstrated by thin-section EM and freeze-fracture techniques. In the longitudinal layer of the intestine of dog, gap junctions could not be demonstrated using these two techniques, despite presence of coupling between the cells.

In the guinea-pig taenia coli, several investigators reported presence of gap junctions between smooth muscle cells. Geisweind and Wermbter (1974) observed gap junctions in freeze-cleaved replicas of guinea-pig taenia coli. However, Gabella (1975) could not demonstrate gap junctions in this tissue.

The presence of gap junctions in the bovine tracheal smooth muscle was reported by Cameron and Kirkpatrick (1977). An examination of their published electron micrograph reveals close contacts which cannot be identified satisfactorily as gap junctions. There was no high power micrograph of a gap junction in their report.

In the canine tracheal smooth muscle, Suzuki et al (1976) claimed that cellular connections like the "tight junction (nexus)" were rare.

In their study on the incidence of nexal contacts and electrical coupling in the guinea-pig and rat vasa deferentia, Goto et al (1977) reported a correlation between presence of nexal contacts and cell-to-cell coupling. They observed a denser innervation in the rat vas deferens than in the guinea-pig tissue. The incidence of "complex nexuses" (projection of one cell into another) were found to be higher in the guinea-pig tissue than in the rat tissue. The guinea-pig tissue had a longer space constant (about 1.5 mm) than the rat tissue (less than 0.5 mm). The longer space constant in the guinea-pig vas deferens was attributed to the presence of greater number of "complex nexuses" thus allowing for better coupling. The higher density of innervation in the rat vas deferens was taken as evidence to support the model proposed by Burnstock (1970) for classifying smooth muscle based on the nature of innervation. The tissues in this study were

were fixed in permanganate which has been shown to result in formation of nexus-like structures in a variety of tissues (see earlier section on nexus). Thus, their evidence for a role of "complex nexuses" in cell-to-cell coupling should be interpreted with caution.

The cells in smooth muscles like guinea-pig vas deferens and taenia coli and the tracheal smooth muscles of bovine and canine species are known to be electrically coupled and have cable properties (Tomita, 1966 and 1967 and Abe and Tomita, 1968 and Cameron and Kirkpatrick, 1977 and Kroeger and Stephens, 1975 and Suzuki et al, 1976). Burnstock (1970) proposed that the extent of electrotonic coupling and the density of innervation of smooth muscles were inversely related, i.e. smooth muscles where electrotonic spread of spike activity did not occur had a very high density of innervation. However, this was shown not to be true of some smooth muscles (see Cameron and Kirkpatrick, 1977).

If smooth muscles exhibit cable properties, what are the morphological correlates of coupling between cells? This is discussed in the following section.

The passive electrical properties of smooth muscle and cable theory:

The passive electrical properties of the smooth muscle cell membrane are the membrane potential, membrane resistance, membrane capacitance, time constant, and the length constant of the membrane. Different electrical models are available to interpret various attempts to measure these characteristic constants: the leaky condenser model and cable models: one dimensional, two-dimensional and cable model for limited length. The one-dimensional cable model has been applied to smooth muscle using the insulated partition stimulating method (Tomita, 1966 and Abe and Tomita, 1968).

For an excitable tissue to have cable properties, it must meet the following criteria (Hodgkin and Rushton, 1946):

i) There should be exponential decay of electrotonic potential along the tissue. The electrotonic potential during steady state at distance x is expressed as:

$$V\ (X,\ t = \infty) = V\ (X = 0,\ t = \infty)\ e^{-X/\lambda} \qquad (1.)$$

The amplitude of the electrotonic potential at $X = 0$, $V\ (X = \lambda)$, can be expressed as $V\ (X = 0,\ t = \infty)/e$. In other words, the space constant can be measured graphically at a distance at which the electrotonic potential decays to 1/e or 37%.

ii) The time course of the electrotonic potential, predicted from the cable equation must fit the actual time course of the elicited electrotonic potential measured at X;

iii) The time to reach half-amplitude of the electrotonic potential increases linearly with distance along the cable. The slope of this relationship is given by $\tau_m /2\lambda$, where τ_m is the membrane time constant; and

iv) The foot of a propagating spike rises exponentially and its time constant τ_f is determined by the cable properties of the fibre.

The above-mentioned criteria are fulfilled by many smooth muscles (Tomita, 1970 ; Kuriyama, 1970). In the partition stimulating method, the tissue is stimulated extracellularly and the evoked potentials are recorded intracellularly at various distances along the muscle from the site of stimulation. The smooth muscle is treated as a series of independent cables formed by end-to-end connections of cells. The potential change in cells in the radial and circumferential axes is considered similar and it decreases along the length only. The maximum variation in the evoked potentials in the radial direction has been calculated to be about 20% (Bennett, 1972). Bennett also points out the uncertainty as to what extent this arises from variations in the quality of impalements of cells and an actual variation in the potential. Eisenberg and Johnson (1970) have critically reviewed this aspect for the analysis of the cable properties.

The passive membrane properties of visceral smooth muscles have been measured with the partition stimulating method using the one-dimensional cable model.

The equation relating space constant λ is as follows:

$$\lambda = (1/4\ D\ R_m/R_i)^{1/2} \qquad (2)$$

where D is the fibre diameter and R_i is the longitudinal resistance of the muscle fibre. To measure R_m, λ and R_i must be known. Alternatively, R_m can be calculated from the relationship $T_m = C_m\ R_m$; where T_m is the time constant and C is the capacitance. The membrane time constant is calculated experimentally from analysis of electrotonic potentials produced by square current pulses.

In the intercalated disc of canine myocardium, where the longitudinal core of the fibre is interrupted by the discs, Spira (1971) has described a modification to the cable equation for determining junctional resistance as follows:

$$\lambda = \sqrt{\frac{r_m}{r_i}} \qquad (3)$$

In cardiac muscle, $r_i = r_{cytoplasm} + r_{disc.}$. For smooth muscle, this would be $r_i = r_{cytoplasm} + r_{nexus}$. The resistances are further given by:

$$r_m = \frac{R_m}{2\ \pi a}\ ,\quad r_i = \frac{R_i}{\pi\ r^2}\quad \text{and}\quad r_n = \frac{n\ R_n}{\pi\ a^2} \qquad (4)$$

R_m = Specific membrane resistance in ohms Cm^2.

R_i = Internal fibre resistivity on ohms Cm.

R_n = Specific nexal resistance in ohms Cm^2.

n = Number of nexuses (discs) per cm length of fibre.

a = Fibre radius.

$$r_n = \frac{nR_N}{\text{area of nexus for fibre}} \qquad (5)$$

The area of the nexus is taken as some fraction (f) of the cross-sectional area of the fibre. Thus,

$$\lambda = \sqrt{\frac{\dfrac{R_m}{2\ \pi\ a}}{\dfrac{R_i}{\pi\ a^2} + \dfrac{nR_N}{f\ \pi\ a^2}}} \quad \text{or} \quad \lambda = \sqrt{\frac{R_m a}{2\ \ R_i + \dfrac{nR_N}{f}}} \qquad (6)$$

f can be calculated from the relationship:

$$f = \frac{\text{nexal length}}{\text{fibre width}} \qquad (7)$$

In order to calculate R_n, values of f, R_m, R_i and a must be known. In the cardiac intercalated disc, Spira determined R_n to be 1.4 ohms Cm^2. In smooth muscle, the nexal resistance can be calculated from the above-mentioned relationship. The values mentioned below (for guinea-pig taenia coli) were taken from Kuriyama and Ito (1975) and Burnstock (1970).

$R_m = 60\ K\,\Omega\ Cm^2$ (range 30 - 90 $K\,\Omega\ Cm^2$).

Radius of fibre, $a \cong 3\ \mu m$.

Fibre length = 100 μm.

Number of fibres/ cm length (n) = 100.

Nexal length = 0.15 μm (measured in canine trachealis, Chapter II).

Therefore $f = \frac{0.15}{6} = 0.025$.

R_N was calculated to be $\underline{5\ \Omega\ Cm^2}$.

The assumptions implicit in these calculations were essentially the same as for the intercalated discs of canine myocardium (Spira, 1971). They are:

All current passes through the nexus and that current passes directly between the cells, i.e., there can be no medium capable of shunting the current to the extracellular medium. The latter assumption was made by Spira (1971) on the premise that the membranes at the nexus fused. Later studies, however, showed the nexus to have a central gap. It is conceivable that the membranes at a gap junction come into contact at discrete regions maintaining direct cytoplasmic continuity between the coupled cells.

From the equation describing the space constant it is evident that changes in any of the three parameters: R_m, R_i and/or R_n will affect λ (Equation #6). Thus, an increase in R_m and/or a decrease in either R_i or R_n will increase λ. In situations where an alteration of λ is observed experimentally, the interpretation should take into consideration possible changes in all the three parameters. Even if R_m and R_i remained unchanged, changes in space constant would be realized by changes in R_n. Among the factors which could change R_n are: presence of "low-resistance" contacts of any type and their number and distribution in smooth muscles.

Regulation of gap junction formation:

Formation of gap junctions *in vitro* has been studied in various cell systems. The techniques used in most cases involved either separation of the cells mechanically in low calcium media or using protroteolytic enzymes. Alternatively, the dissociated cells were either reaggregated in culture or micromanipulated into contact. Presence of gap junctions was studied in thin-sections and/or by the freeze-fracture techniques. Electrical coupling between the cells was measured using electrophysiological techniques. *In situ* formation of gap junctions has also been investigated in a number of tissues. The cells and tissues investigated and various factors determining the appearance of gap junctions and coupling are discussed in Chapter IV (Discussion section).

Potassium conductance blockers:

Multiunit nature of canine tracheal smooth muscle:

The canine tracheal smooth muscle has been described as belonging to the multiunit type (Kroeger and Stephens, 1975). The types of innervation found in this smooth muscle were described earlier (section on innervation of smooth muscles). Gap junctions have not been convincingly demonstrated in this tissue. The muscle was shown not to exhibit spontaneous electrical and mechanical activities *in vitro*. The electrophysiological studies reported in literature (Kroeger and Stephens, 1975 and Suzuki *et al*, 1976) showed that tetraethylammonium (TEA) could bring about a conversion from multiunit to single-unit type of behaviour of this smooth muscle *in vitro*.

The compound TEA (a quaternary ammonium ion) has been used as a tool to block potassium channels of excitable tissues (see Narahashi, 1974). Voltage-clamp studies demonstrated that the quaternary ammonium ion TEA selectively inhibited potassium conductance. Tasaki and Hagiwara (1957) first studied the effect of TEA on membrane conductances in the squid giant axon. TEA prolonged the falling phase of the action potential, creating a plateau phase much like the cardiac action potential. It was effective only when perfused internally. Voltage-clamp studies by other investigators were interpreted as confirming the K^+ - conductance blocking effect of TEA (see Narahashi, 1974).

TEA has been shown also to decrease the resting potassium conductance of other tissues; e.g., in skeletal muscle membranes (Stanfield, 1970 and Volle, 1970 and Volle *et al*, 1972). In Aplysia neurones, TEA (when applied internally) prolonged the duration of action potentials (IPSP) (Kehoe, 1969 and 1972). These effects were described as resulting from block of potassium conductance. When TEA was externally applied, the block of IPSP required a concentration 500 times lower than that needed to block from internally. The external effect was interpreted as being due to block of acetylcholine receptors. TEA has been used to eliminate the steady-state current during depolarization in studies to elucidate the relationship between depolarization and release of transmitter (Katz and Miledi, 1967, 1969 and 1971).

The aminopyridines have been used recently as tools to block potassium channels in excitable membranes. The compound 4-aminopyridine (4-AP) has been reported to selectively block the potassium channels of axons of cockroach (Pelhate and Pichon, 1974); squid (Meves and Pichon, 1975 and Yeh et al, 1976); Myxicola (Schauf et al, 1976); and the frog node of Ranvier (Wagner and Ulbricht, 1975). The selectivity of action of 4-AP in blocking K^+ conductance was evident from these studies. However, Yeh et al (1976) while investigating the effects of this compound in squid axon membrane concluded that, in addition to being selective, it exhibited voltage-, time-, and frequency-dependent characteristics. They also showed differences in the mechanisms of action of TEA and 4-AP in the squid axon (discussed in Chapter III, Discussion).

The effects of 4-AP on smooth muscle have not been investigated thus far. TEA has been known to convert the normally multiunit canine trachealis to unitary behaviour. In my studies (reported in Chapter III), evidence will be presented that 4-AP is also capable of bringing about this conversion.

B. Objectives of the Present Study

The following questions were raised at the start of this investigation:

i) Can gap junctions be demonstrated in this smooth muscle and, if so, are they necessary for cell-to-cell coupling in multiunit smooth muscles?

ii) What are the structural bases of the conversion from multiunit to single-unit type of behaviour of canine tracheal smooth muscle, if any?

iii) Can all K^+ conductance blockers bring about this conversion, both functionally and structurally?

CHAPTER II

NATURE OF CANINE TRACHEAL SMOOTH MUSCLE AND EFFECTS OF TETRAETHYLAMMONIUM ION (TEA) ON CONTRACTILE FUNCTION AND STRUCTURE.

CHAPTER II

Introduction: Nature of Tracheal Smooth Muscle

Each cell of a multiunit smooth muscle must be excited independently either by excitation of nerves or by diffusion of mediator through the interstitial space. Gap junctions and any other regions of close contact providing low-resistance coupling between smooth muscle cells are absent or ineffective in these tissues according to the original classification (Bozler, 1948). Canine tracheal smooth muscle has been described as a multiunit smooth muscle and shown to have no spontaneous electrical and mechanical activities as studied in vitro (Kroeger and Stephens, 1975 and Suzuki et al, 1976). The contractile functions of respiratory smooth muscle are thought to be regulated by both the parasympathetic and the sympathetic components of the autonomic nervous system (Widdicombe, 1963). The cholinergic innervation from the vagus is excitatory (Widdicombe, 1963) while the adrenergic is inhibitory (Foster, 1964 and Rikimaru and Sudoh, 1971). In the canine tracheal smooth muscle, agonists acting on α-adrenergic receptors excite and those acting on β-adrenergic receptors relax (Suzuki et al, 1976). There is evidence of a non-adrenergic and non-cholinergic inhibitory innervation in the tracheobronchial smooth muscle of the guinea pig (Coburn and Tomita, 1973; Bando et al, 1973; Coleman and Levy, 1974; and Richardson and Bouchard, 1975) and man (Richardson and Beland, 1976) similar in some ways to the one proposed for the intestinal smooth muscle (Burnstock, 1970). The similarity in the neural control of the respiratory smooth muscle to that of the gastrointestinal tract smooth muscle might be expected from the fact that embryologically these two systems have a common origin (Krahl, 1964).

Studies on the density of innervation of the airway smooth muscle of the bovine species indicate a sparse distribution of nerves (Cameron and Kirkpatrick, 1977). The axons and their varicosities were noted mainly in the clefts between the smooth muscle cell bundles. These morphological findings do not correspond to expectation for a multiunit smooth muscle since each cell would have to be excited directly.

Reported effects of TEA on the canine tracheal smooth muscle:

The smooth muscle cells of the canine trachea had a stable membrane potential in vitro with no evidence of spontaneous oscillations or action potentials. The membrane had marked rectifying property to depolarizing stimuli. Neither electrical stimulation nor application of a variety of stimulatory agents caused phasic electrical or mechanical responses (Kroeger and Stephens, 1975). A myogenic response to quick stretch, normally present in single-unit smooth muscles, was not found. Treatment in vitro with TEA however brought about a conversion from multiunit to single-unit type of behaviour. TEA depolarized the cells, abolished the rectification, and initiated phasic mechanical activity with action potentials. A myogenic response to stretch could then be demonstrated (Kroeger and Stephens, 1975).

In electrophysiological studies, Kroeger and Stephens (1975) found that TEA increased the amplitude of the electrotonic potentials resulting from hyperpolarizing stimuli. The space constant of the tissue was also increased from 1.6 to 2.8 mm after TEA treatment. Since the magnitude of the electrotonic potential is related to the membrane resistance (Abe and Tomita, 1968 and Abe, 1970), these effects of TEA on the space constant were interpreted as being the result of an increase in the transmembrane resistance. However, the authors assumed that the electrical coupling and internal core resistance remained unchanged after TEA treatment.

The effects of TEA were also interpreted as being consistent with its known action, namely, blocking potassium conductance. Kroeger _et al_ (1975) concluded that the resting conductance to K^+ in this tissue was so high and the increase in Na^+ conductance so small as to prevent a regenerative increase in Na^+ permeability resulting in depolarization and spontaneous firing of pace-maker cells.

Gap junctions have been considered to play a role in cell-to-cell electrical coupling in smooth muscle as well as in a variety of other excitable tissues (Griepp and Revel,1977), although such evidence is largely circumstantial in this tissue. Since TEA treatment results in an increase in the space constant of canine tracheal smooth muscle tissue, I considered that this could be the result of not only the observed increase in the transmembrane resistance, but also to a possible increase in the gap junctional area.

Objectives

The objectives of the present study were:

i) To investigate the types of innervation and their relationship to smooth muscle cells and the effects of nerve stimulation on the mechanical properties of the muscle as studied in vitro;

ii) To study the incidence of gap junctions between smooth muscle cells of the canine trachea and changes in their number in tissues exposed to TEA when single-unit behaviour is established and thus to determine whether changes in junctional resistance might account for the observed alterations in electrical and mechanical properties;

iii) To study the correlation between gap junction formation and mechanical activity with respect to time, TEA concentration and other variables.

Materials and Methods

Tissue preparation: Mongrel dogs were anaesthetized with pentobarbital sodium (30 mg/Kg, i.v.) and the cervical tracheae were isolated by a ventral midline incision. The tracheal smooth muscle (Trachealis) which lies on the dorsal aspect of the trachea, bridging the defect in the cartilaginous rings, was isolated gently and carefully from the underlying paries membranaceous and cut into strips 1 cm x 0.2 cm x 0.075 cm. The tissue strips were mounted vertically in a 20-ml organ-bath at 37° C containing Krebs-Ringer bicarbonate solution (Krebs solution) through which a mixture of 95% O_2 - 5% CO_2 was bubbled continuously. The tissues were allowed to recover from dissection and handling for a period of one hour before addition of drugs or application of field stimulation. One end of the muscle strip was attached to the tube holding the electrodes for field stimulation and the other end to a Grass FT-03 force transducer. Isometric tension was recorded in a Beckman R 611 recorder through an appropriate coupler. An initial one g tension was applied to the tissue strips.

Solutions: The composition of the Krebs solution used is given in Table I: Solutions containing TEA were prepared by replacing equimolar amounts of NaCl by TEA from the Krebs solution.

Electrical stimulation: Electrical stimulation was applied through a pair of electrodes one cm apart and placed concentrically around the tissue strip. The current was applied by a Grass S9 stimulator. The parameters chosen for nerve and direct muscle stimulations are given in legends to Figures.

Electron microscopy (EM): At the end of the incubation or experimental period, the tissue strips were fixed by immersion in 2% glutaraldehyde in 0.075 M cacodylate buffer (pH 7.4) containing 4.5% sucrose and 1 mM $CaCl_2$ under isometric conditions. After 2 hours in fixative, the tissues were rinsed for one hour in 0.1 M cacodylate buffer (pH 7.4) and post-fixed in 1% osmium tetroxide in 0.05 M cacodylate buffer of the same pH for 90 minutes. All tissues were stained en bloc with saturated aqueous uranyl acetate for one hour, dehydrated in graded alcohols and embedded in Spurr resin. Tissues were oriented parallel to the transverse axis in the blocks so as to get cross-sections of smooth muscle cells. Thin sections cut on a Porter-Blum MT-2B Ultramicrotome with glass knives and mounted on

TABLE I

Composition of Krebs Solution

Salts	Normal Krebs-Ringer (mmoles/litre)
NaCl	121.9
KCl	4.7
$CaCl_2$	2.5
$MgCl_2$	1.2
KH_2PO_4	1.2
$NaHCO_3$	15.5
Glucose	11.0

300-mesh copper grids were stained for 2 minutes with Lead citrate and examined in Jem-7A or Philips-301 electron microscopes.

Mechanical studies: Tissues were incubated either in Krebs solution or in TEA-containing solutions and isometric tension was monitored.

Dose-response relationship: The effects of various concentrations of TEA (from 1 mM up to 70 mM) on the phasic mechanical activity of canine trachealis were studied. From this relationship, four concentrations, 3, 10, 20 and 33 mM, of TEA were chosen. The minimum concentration of TEA capable of inducing a mechanical activity was found to be 3 mM. The tissue strips were incubated for one hour and fixed for electron microscopy. Control tissues were incubated in Krebs solution for one hour and processed for electron microscopy.

Time-course of TEA-induced response: The tissue strips were incubated with TEA-containing solutions until phasic mechanical activity was established (10 - 15 minutes) and fixed for EM. Control tissues were incubated in Krebs solution.

Quantitation of gap junctions: The number of gap junctions in thin sections of smooth muscle cells cut transversely were counted at a magnification of 45,000 in 3 to 5 grid-squares from each tissue. Cell-to-cell contacts were counted as nexuses or gap junctions if they presented a 5-lined or a 7-lined structure with a 2 nm central gap. A series of non-overlapping photographs of the cells in the scanned grid squares were taken at a magnification of 3200. The negatives were enlarged 3 times (x 9600 final magnification) and printed on 20.3 x 25.4 cm paper. A map measurer (Keuffel and Esser Co., Switzerland) was used to measure the circumferences of cells in the photographs.

In order to ensure that nexuses were not counted from serial sections of tissues, the measurements were made from grid squares occupied by the same secti[illegible] The number of such grid squares depended on the section dimensions and so large sections of tissues were made. The number of gap junctions counted in the scanned grid squares was expressed as number per 1000 μm length of membrane.

The tissue blocks were assigned different codes. The codes were broken only after the number of gap junctions were determined as well as the total membrane length measured.

The diameter of the gap junctions counted in the scanned grid-squares was measured from high magnification micrographs of cells from control and TEA-treated tissues.

Statistics: The Kruskal-Wallis H test was used for comparing the significance of the mean number of gap junctions in the control and the TEA-treated groups and the null hypothesis was rejected if $p\ (H \geq H')$ at 0.05 level. Pairwise comparisons of the data were made using the Mann-Whitney U test.

Results

A. General Ultrastructure:

Freshly excised tracheal smooth muscle tissues from the dogs were fixed in 2% glutaraldehyde in 0.01 M cacodylate buffer (pH 7.4) and processed for electron microscopy as described in the Methods section. Grey to silver sections of tissues, where the cells were cut in transverse orientation, were examined under the electron microscope.

Figure 1 shows a low-magnification view of tracheal smooth muscle bundle. The bundles are separated by large interstitial spaces containing bundles of collagen and fibroblasts.

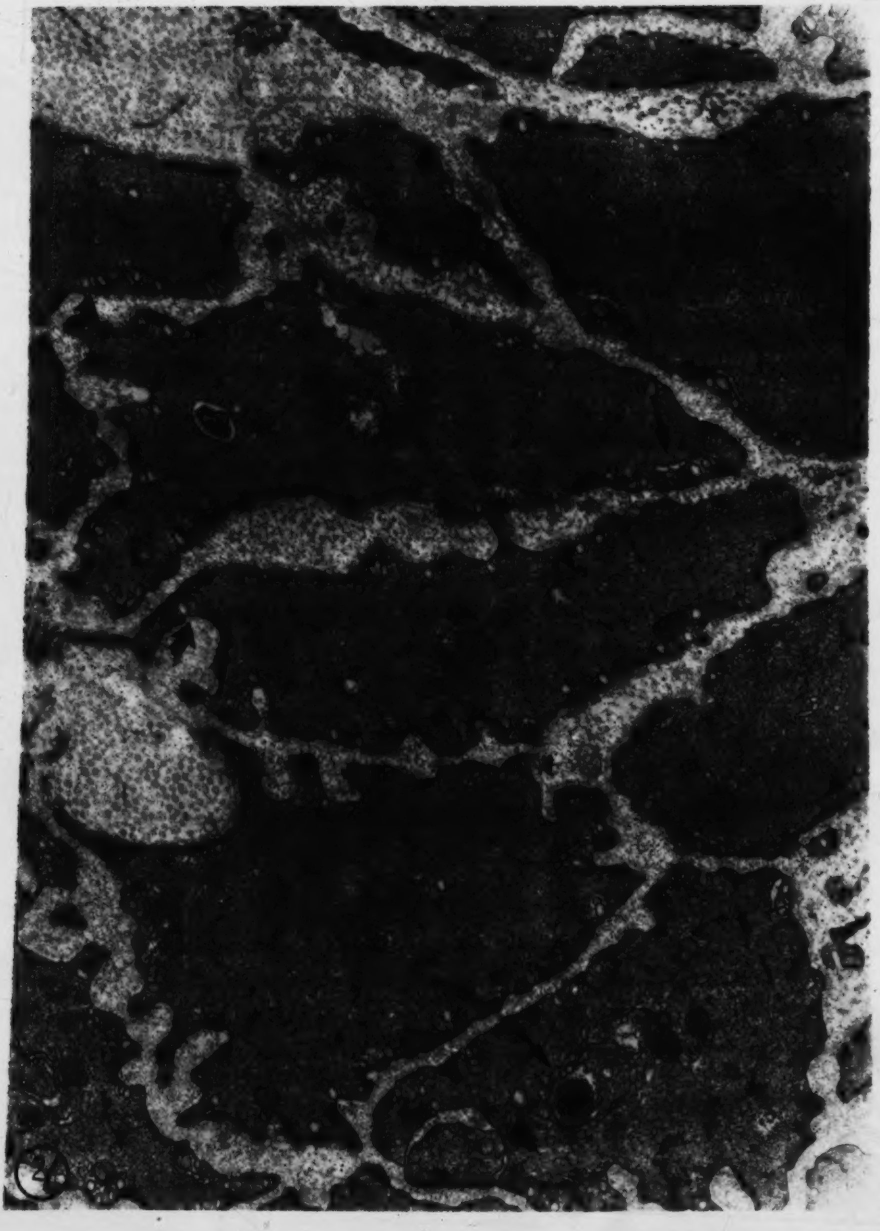
Figure 2 shows a higher magnification view of a few smooth muscle cells. The cells are very irregular in shape, with several processes. These processes were seen in both control tissues as well as those treated with TEA. These processes did not appear to be those which result from contraction of smooth muscle as described by Gabella (1976). In the guinea-pig taenia coli, that author has shown changes in the structure of smooth muscle cells upon contraction and relaxation. Tissues fixed in the shortened state show an increase in the cross-sectional area. Individual muscle cells show similar changes. The cells have a very irregular surface with large evaginations. On the other hand, in elongated tissues, the cell surface is smooth. The tracheal smooth muscle strips used in this study were fixed with an initial one gm load and can therefore be considered stretched. Also, they were unable to relax in response to various agents and showed no evidence of tone or stable active tension. Nevertheless, the cells do not appear to have smooth surfaces. The numerous projections of the cells seen in this tissue couldn't have been an artefact of shortening either from an effect of the fixative or from spontaneous contractions induced by TEA _in vitro_.

Examination of thin sections of tissues revealed presence of gap

Figure 1. Smooth muscle cell bundle from the canine trachealis. Bundles of collagen and a few fibroblasts (F) can be seen in the space outside of the smooth muscle cell bundle. Magnification x 6400.

1

Figure 2. Canine tracheal smooth muscle cut in cross-section. The cells have numerous projections and a gap junction (arrow-head) can be seen. The caveolae (small arrows) appear along the surfaces of the cells. Smooth sarcoplasmic reticulum (SR) can be seen (arrows) close to mitochondria (M) and underneath the caveolae. The mitochondria are in orthodox form. The myofilaments and dense-bodies are seen in the cytoplasm. Magnification x 74,000.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junctions between smooth muscle cells. These junctions were seen connecting processes of two cells. Figures 3 and 4 show typical gap junctions as seen in thin sections. The structure is 7-layered with a central gap about 2 nm wide. The abutment type of gap junctions described in other smooth muscles (Henderson et al, 1971) were never observed in the canine trachealis.

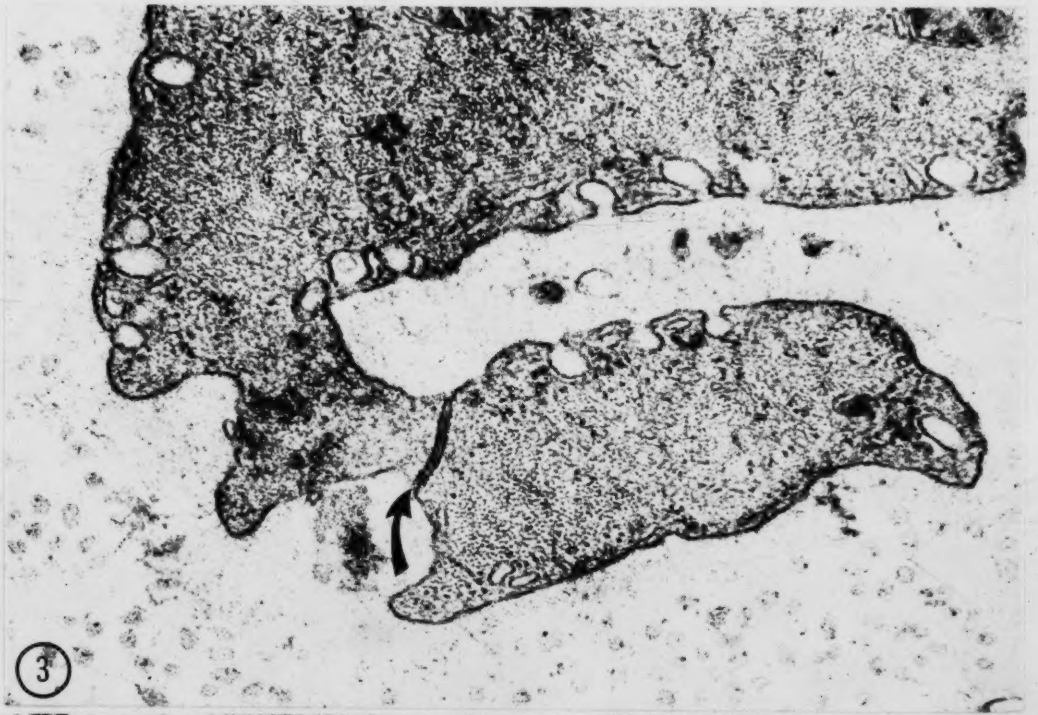
The fixation of the tissues was found to be optimal judging from the appearance of intracellular organelles and other criteria. The mitochondria appeared orthodox. The sarcoplasmic reticulum (SR) was of two types: smooth and rough with attached ribosomes. The smooth SR was found usually close to mitochondria as well as the surface membrane (Figure 2). The myofilaments were well preserved and were of three types: thin filaments measuring about 70 A° in diameter; thick filaments measuring about 150 A° in diameter; and intermediate filaments about 100 A° in diameter. There were many dense-bodies in the cytoplasm and dense areas subadjacent to the plasma membrane were found in regions lacking caveolae. The Golgi apparatus with stacked cisternae were seen occasionally.

The surface membrane had many caveolae (Figures 2 and 3). Many of the caveolae could be seen to open into the extracellular space. Some of them were also found inside the cytoplasm with no apparent opening into the extracellular space. This might be due to the connection being out of the plane of section or their presence intracellularly. The plasma membrane had the typical three-layered structure approximately 80 A° in thickness.

Nerves: The intervening space between bundles of smooth muscle cells had many fibroblasts and collagen bundles. The autonomic nerves were

Figure 3. A gap junction (arrow) between processes of two smooth muscle cells. Magnification x 74,000.

Figure 4. A gap junction between two smooth muscle cell processes at a higher magnification. The 7-layered structure with a central gap (small arrows) is evident. Magnification x 147,000.



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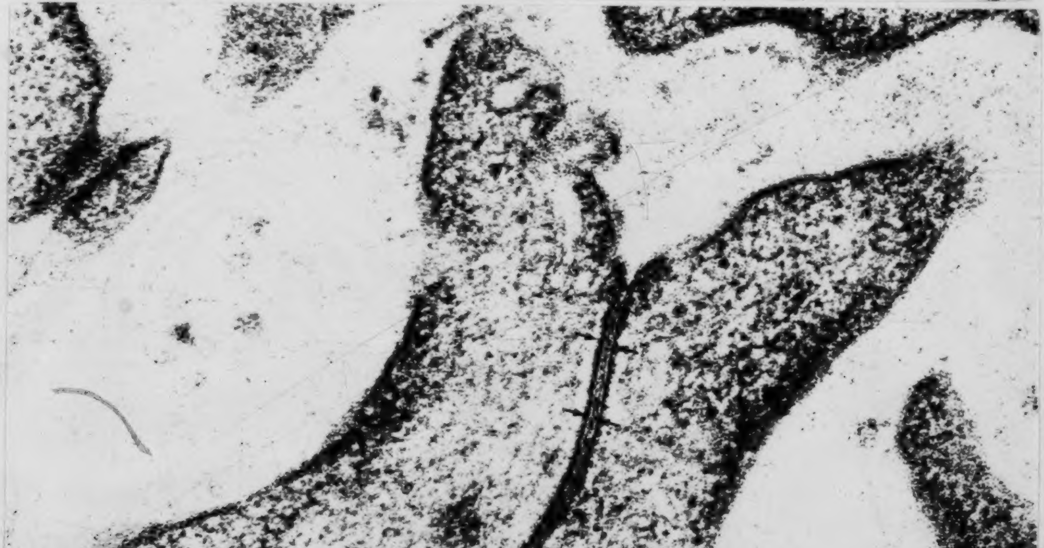


④

seen in this space. Figure 4 a shows such nerve bundles in the tracheal smooth muscle of the dog. Typically these bundles consisted of axons as well as the axonal varicosities ensheathed by Schwann cell cytoplasm. At some regions, the axonal varicosities were not fully enclosed in or were even devoid of the Schwann cell sheath (Figure 4 b). Occasionally, nerve bundles or several axons were observed within muscle bundles (Figures 5 a and b). The closest distance of the varicosity from the smooth muscle cell observed was 1400 A°.

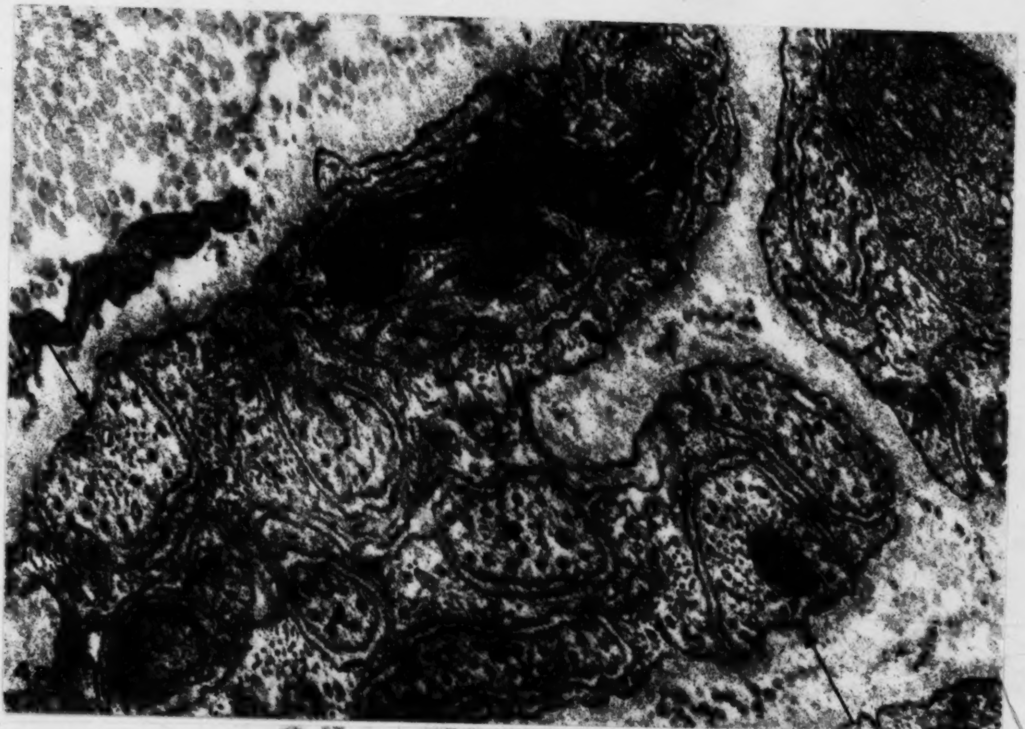
The varicosities of the nerves contained three types of vesicles (Figures 4 b, 5 b and 6), a) small agranular vesicles about 500 - 700 A° wide; b) small granular vesicles about 600 - 800 A° wide; and c) large granular vesicles about 1000 - 1200 A° wide. Occasionally varicosities were seen which could not be classified. Amongst these, the agranular vesicles were the most abundant. Suzuki _et al_ (1976) have demonstrated with histochemical methods, the presence of acetylcholinesterase and catecholamine-positive nerve fibres in the canine trachealis.

B. Mechanical response to field stimulation:

The effects of electrical field stimulation on the motor responses of canine trachealis were studied. Field stimulation was applied at 10 Hz, 40 V and 0.5 msec pulse duration to stimulate the intrinsic nerve endings. To stimulate muscle directly, 50 Hz, 15 V and 5 msec pulse duration were used. The amplitude of the pulse was varied over a wide range, keeping the duration and frequency constant, Figure 4 a. The axons (A) are ensheathed in Schwann cell (S) cytoplasm. Some of the axons (arrows) are not completely enclosed by the Schwann cell cytoplasm. Magnification x 68,000.

Figure 4 b. Varicosities in the canine trachealis. One varicosity (single arrow) has small agranular vesicles (SAV). Two varicosities (double arrows) in the profile have both SAV and large granular vesicles (LGV). Magnification x 50,000.

Abbreviations SAV and LGV will be used in Figures 5 a, 5 b and 6.



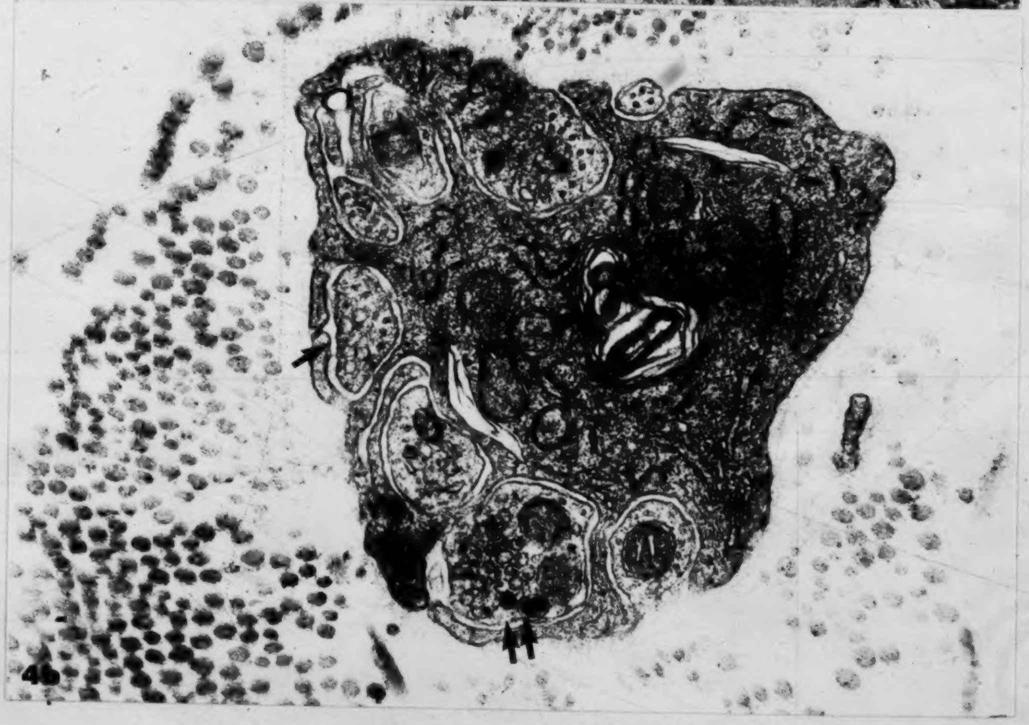
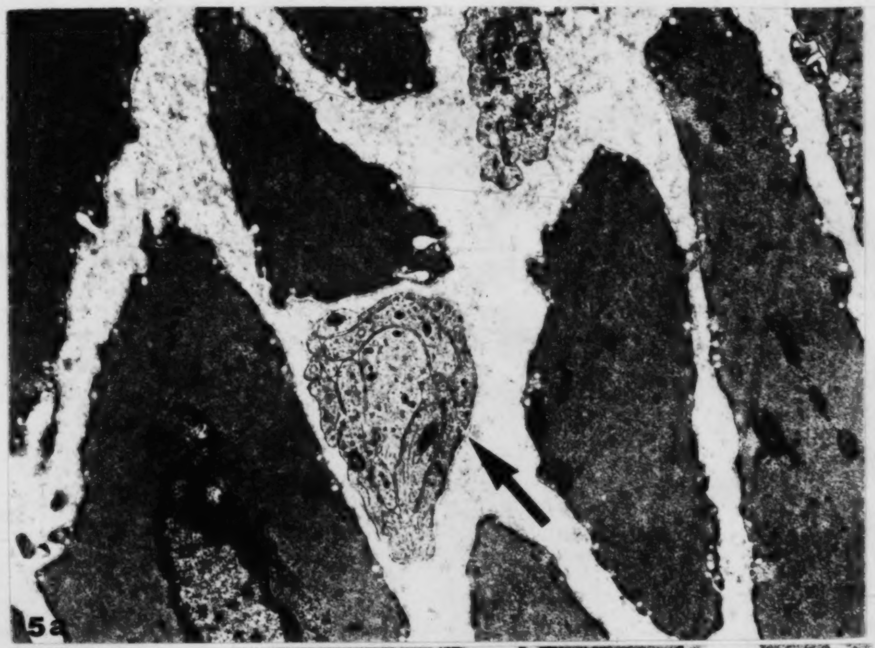

4b

Figure 5 a. A bundle of axons ensheathed in Schwann cell cytoplasm (large arrow) can be seen within the smooth muscle cell bundle from canine trachealis. Magnification x 40,000.

Figure 5 b. A varicosity (large arrow) containing both SAV and LGV is seen within the smooth muscle cell bundle. Magnification x 51,000.


5a

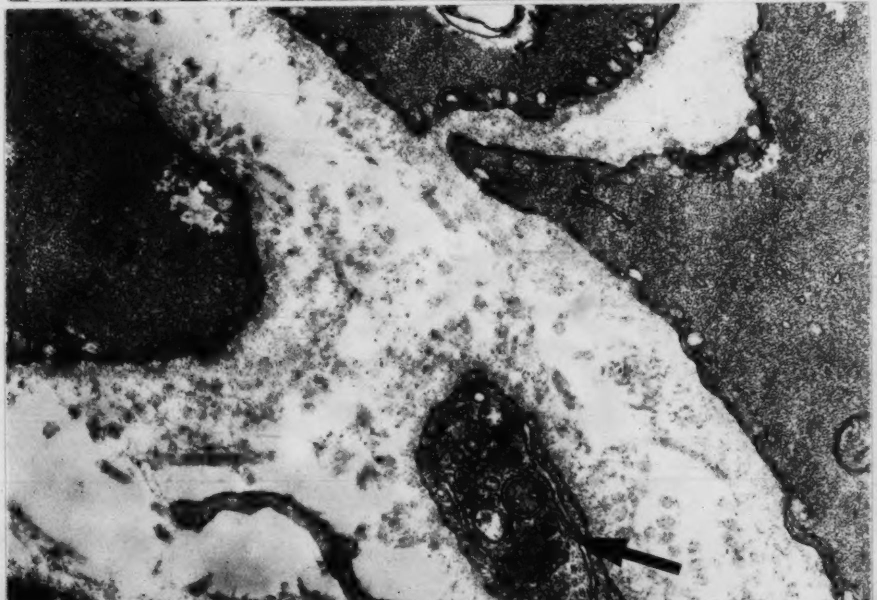
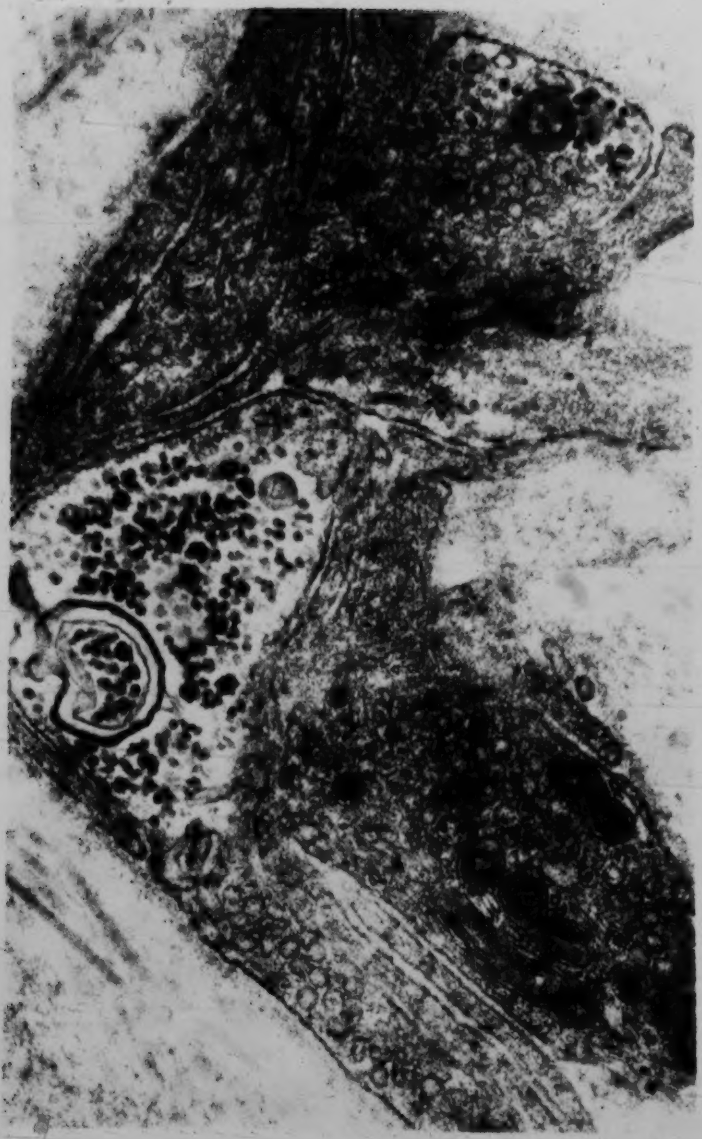


Figure 6. Axonal varicosities in the canine trachealis containing both SAV and LGV. Magnification x 54,000.



⑥

and a 15 V pulse was found optimal for direct muscle stimulation.

Using parameters for nerve stimulation, field stimulation caused an "on"-contraction; the tissue relaxed to the base-line on turning the stimulus off. This contractile response was completely blocked by atropine (10^{-7}M) consistent with activation of cholinergic nerves by field stimulation. Use of parameters for direct muscle stimulation resulted in a contraction whose amplitude was higher than that achieved through nerve stimulation. Atropine (10^{-7} M) diminished this contractile response, but did not block it. Thus at these stimulus parameters both cholinergic nerves and muscle were stimulated (Figures 7 and 8).

C. Mechanical response to TEA treatment:

Canine tracheal smooth muscle (from ten animals) incubated *in vitro* in Krebs solution was quiescent. Application of TEA caused the tension to rise. There was a lag period between exposure to the drug and development of phasic activity. This was dependent on the concentration of the drug used, shorter for higher concentrations of the drug (from a few seconds up to one minute for 33 mM; from 2 to 5 minutes for 3 and 10 mM TEA). The tissues started to exhibit spontaneous rhythmic oscillations of tension, which attained a steady-state. Figure 9 shows the responses to 3, 20 and 33 mM TEA. Though there was fluctuation of tension, it did not return to the original base-line (Figure 10 a).

The steady-state tension attained was dependent on the concentration of TEA used. The maximum tension achieved after 33 mM TEA was greater than for the other three concentrations. The maximum active tension attained in tissues exposed to 10, 20 and 33 mM TEA are given

Figures 7 and 8. Mechanical response of the canine tracheal smooth muscles to electrical stimulation. Arrows indicate stimulus duration.

N - using parameters for nerve stimulation: 40 V, 0.5 msec and 10 Hz.

M - using parameters for direct muscle stimulation: 15 V, 5 msec. and 60 Hz.

Figure 7 - shows mechanical response to electrical stimulation.

Figure 8 - shows mechanical response to electrical stimulation 2 minutes after exposure of tissues to 10^{-7} M atropine.

Consult text for description of responses.

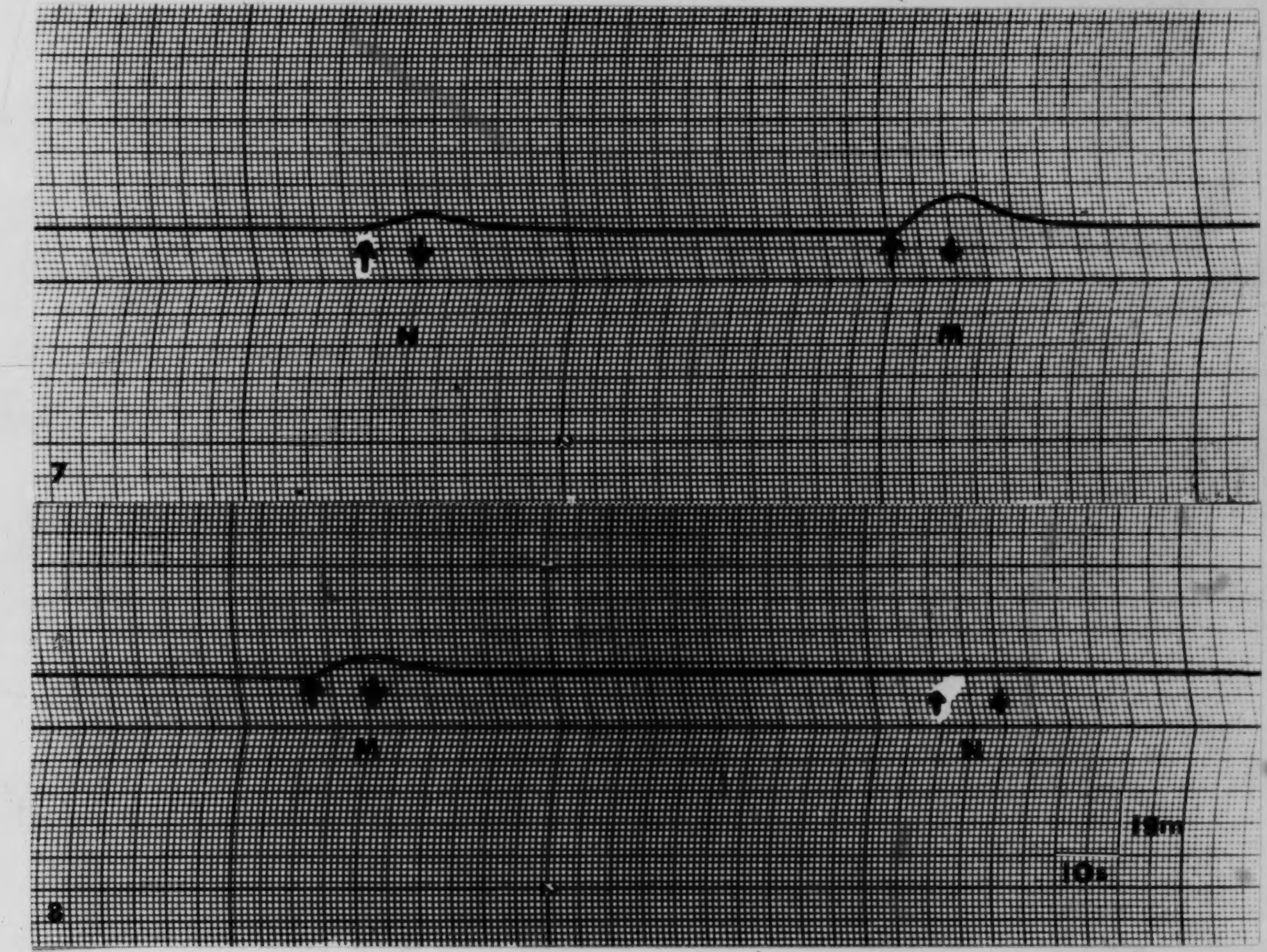

7
N
M
8
M
N
15m
10s

Figure 9. Mechanical activity of canine trachealis strips to 3, 20 and 33 mM TEA. Arrows indicate drug additions. Bottom trace is continuation of 3rd trace (33 mM TEA response). 4 min on top trace indicates time after addition of 3 mM TEA.

Consult text for details.

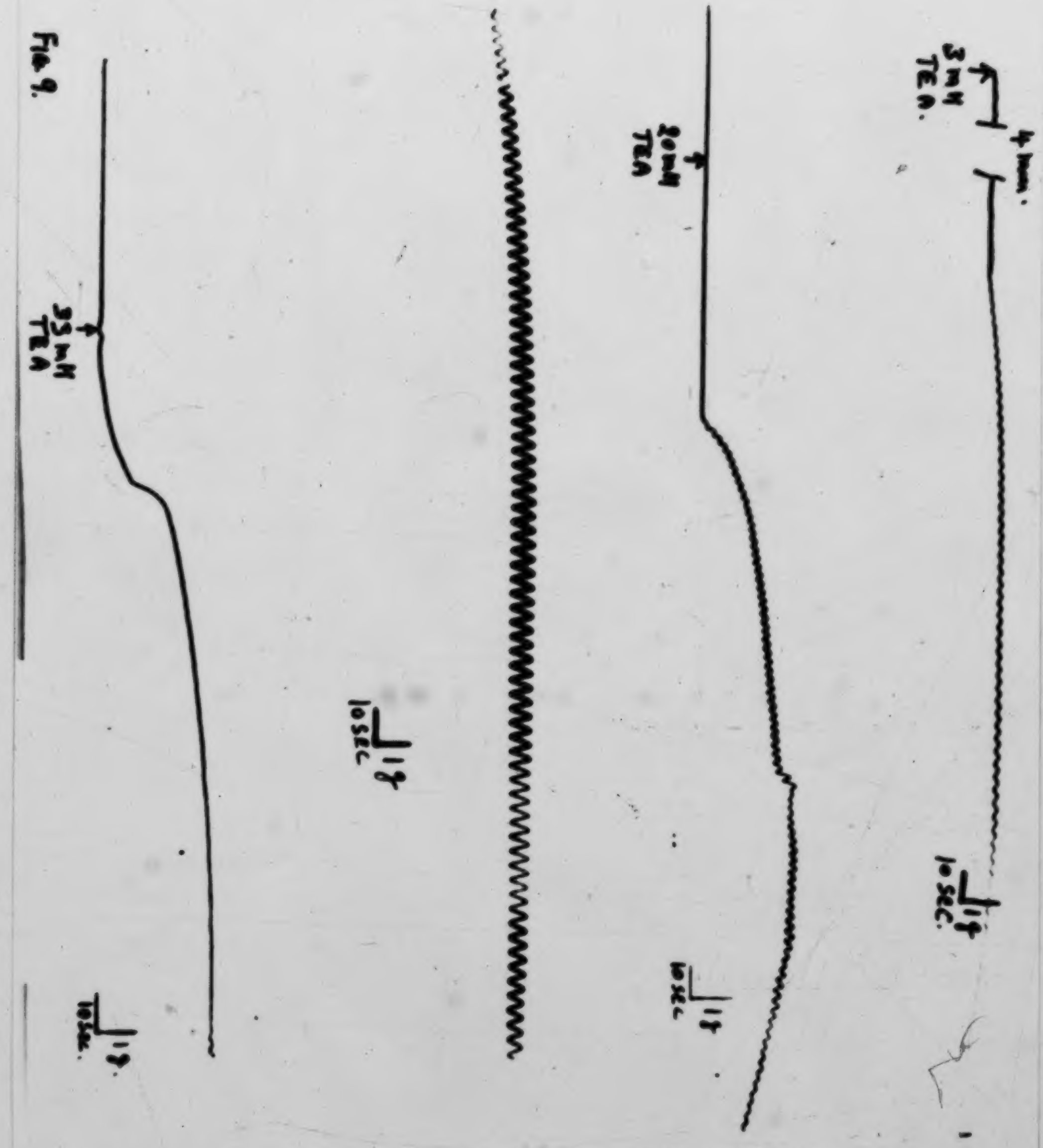

3 mM
TEA.
4 min.
1 g
10 sec.
20 mM
TEA
1 g
10 sec.
1 g
10 sec.
35 mM
TEA
1 g.
10 sec.

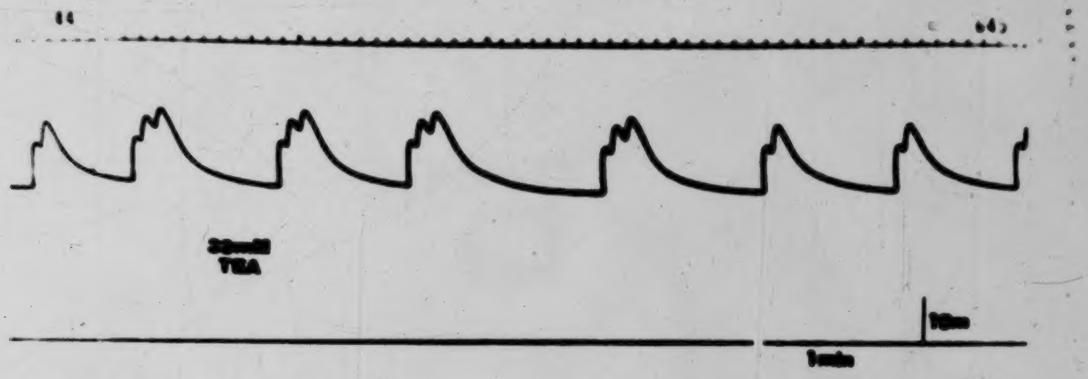
Fig. 9.

Figures 10 a and b.

Figure 10 a. Fluctuation in tension with phasic activity after treatment with 33 mM TEA.

Figure 10 b. Mechanical response to 33 mM TEA after addition of 10^{-6}M atropine (arrow). Note a slight fall in tone.

Solid lines indicate zero tension for both traces. Time and tension calibrations are same for both traces.



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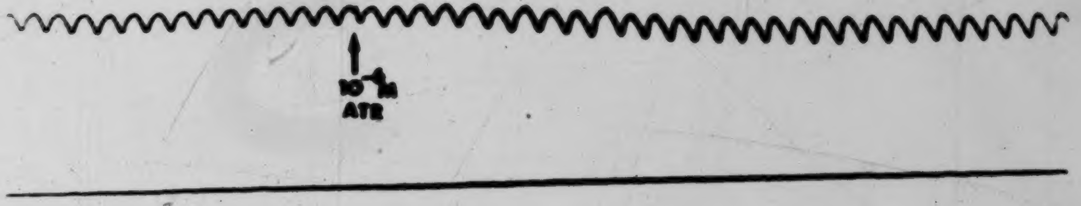


10b

in Table II.

The phasic mechanical activity seen after TEA (3 to 33 mM) treatment was not blocked by atropine at concentrations from 10^{-7} to 10^{-5} M or by TTX (up to 10^{-5} g /ml). However, atropine diminished the tone slightly in these tissues but the tone recovered with time (Figure 10 b). Tissues from animals incubated in Ca^{++}-free Krebs solution containing 0.5 mM EGTA, when later exposed to TEA, did not show any mechanical activity. Mechanical activity could be restored on addition of CA^{++} to the medium (to a concentration of 2.5 mM). D-600 (10^{-5} M), an agent known to block Ca^{++} entry upon depolarization, completely abolished the mechanical activity after TEA treatment. Thus, TEA seems to depend on availability of extracellular Ca^{++} for inducing the mechanical effects. Kroeger et al (1975) have shown the lack of effect of atropine on mechanical activity and the ability of D-600 to block the response. When TEA-containing solutions were replaced with Krebs solution, the tension usually fell sharply to the original base-line. Occasionally, (2 out of 10 cases) the tissues had to be washed with Krebs solution repeatedly to restore tension to base-line after exposure to 33 mM TEA.

D. Effect of field stimulation on TEA-induced mechanical response:

When the phasic mechanical activity was established after application of TEA, the tissues (from six animals) were subjected to electrical field stimulation. The parameters of stimulation are shown in legends to Figures. After treatment with 3 and 10 mM TEA, field stimulation caused an increase in tension. When the tone was maintained at the higher level (after a few seconds), repeating the

TABLE II

Maximum Active Tension in TEA-Treated Tissues[†]

n[††]	10mM	20mM	33mM
10	38.5 (1)*	65 (3)*	100%

[†] The maximum active tension attained in tissues treated with 33mM TEA for one hour was taken as 100% response. This was not the maximum tissue response.

[††] Number of tissues studied.

[*] Values are significantly lower ($p < 0.001$) than maximum response. Response to 10 mM TEA was significantly lower ($p < 0.001$) than the response to 20 mM TEA.

Values in parentheses are ± S.E.

stimulation caused a relaxation (Figure 11). When the tissues were contracted after exposure to 20 mM TEA, field stimulation caused a relaxation. On turning the stimulus off, tension reached the pre-stimulus level (Figure 12). Inability to elicit a contraction at larger doses of TEA could be due to the fact that the tissue was already at near maximal tension and only capable of relaxation. Propranolol (10^{-6} gm/ml) blocked the relaxation to field stimulation in six experiments after TEA treatment and the respone consisted of a contraction (Figure 13), suggesting a β-adrenergic mechanism. TTX (10^{-5} gm/ml) failed to block the relaxation (in 5 out of 6 experiments) nor did it affect the tone. This could mean either operation of a TTX-insensitive mechanism or a relative insensitivity of the tissue to this drug.

E. Gap junctions in control and TEA-treated tissues:

i) Gap junctions in control tissues: Control tissues incubated *in vitro* in Krebs solution and fixed for electron microscopy contained gap junctions. These junctions as seen in thin sections of tissues were always located on cell processes. The number of gap junctions in control tissues was 3.36 ± 0.05 per 1000 μm membrane length (Table III). The measured diameter of the junctions in these tissues was 0.15 ± 0.05 μm. The structure of a typical gap junction seen in thin sections is shown in Figure 3.

ii) Gap junctions in TEA-treated tissues: Four different concentrations of TEA were used in these studies, namely, 3, 10, 20 and 33 mM. Tissues were incubated at 37° C for one hour and fixed for EM.

Figures 11, 12 and 13.

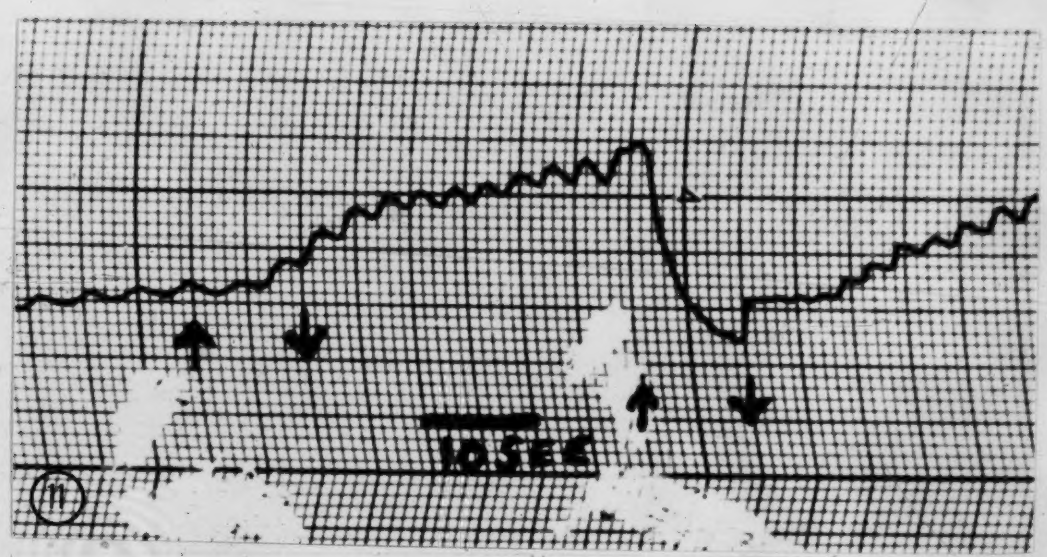
Figure 11. Electrical field stimulation on the TEA-induced mechanical activity of canine trachealis. Arrows indicate stimulus duration. The parameters chosen for stimulation are: 15 V, 5 msec. and 60 Hz.

Figure 12. Mechanical response to electrical stimulation of canine trachealis strip after exposure to 20 mM TEA. Arrows indicate stimulus duration. The stimulus parameters are: 15 V, 5 msec. and 60 Hz.

Consult text for details.

Figure 13. Mechanical response to electrical stimulation of strips treated with 20 mM TEA and 10^{-6} gm/ml propranolol. Stimulus parameters are: 40 V, 0.5 msec. and 10 Hz.

Consult text for description of the responses.



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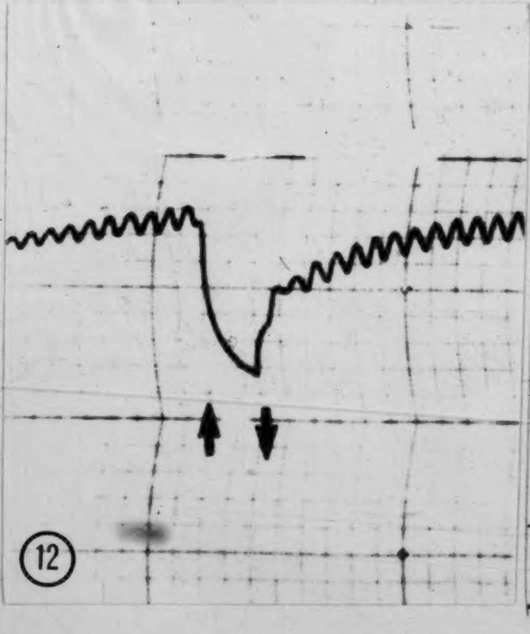

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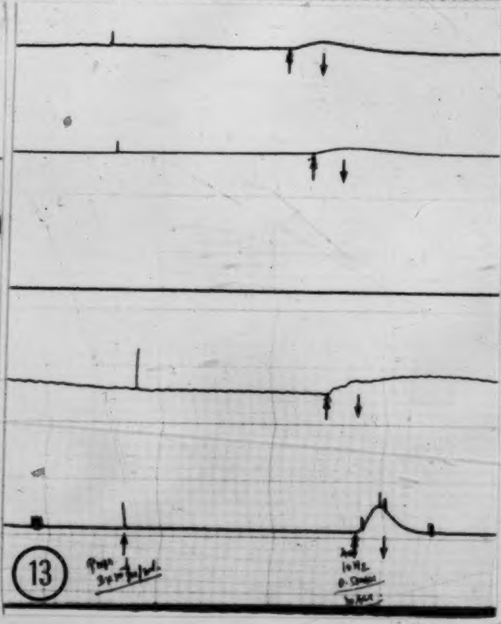


13

The number of gap junctions in these tissues were: 5.35 ± 0.20; 5.04 ± 0.20; 7.4 ± 0.40; and 5.69 ± 0.19 per 1000 μm membrane length in 3 mM, 10 mM, 20 mM and 33 mM TEA-treated tissues respectively (Table III). These values were significantly higher than the number in control tissues (above). However, their diameters (0.17 ± 0.09 μm) were not significantly different from the control value. The number of gap junctions in the various treated groups were not significantly different from each other. Thus, a dose-effect relationship on structure was not evident.

Figure 14 shows the size distribution of the gap junctions measured from micrographs of control and TEA-treated tissues as a percentage of the total number of gap junctions counted from each group. The measured diameters of the gap junctions in control fall in the range of 0.04 to 0.24 μm. However, about 15% of the gap junctions whose diameters were measured from TEA-treated tissues fall in a range of 0.24 to 0.28 μm. The mean diameters from the two groups were not significantly different, as described above. As in control tissues, the gap junctions in the treated tissues were typically found between cell processes.

The number of gap junctions in tissues exposed to 33 mM TEA and fixed at a time when spontaneous mechanical activity was established (10 to 15 minutes) was 5.10 ± 0.16 per 1000 μm membrane length. This value was also significantly higher than the number in control tissues. Moreover, there was no significant difference between this value and the number in one hour-treated tissues. Thus, the structural changes were evident at the time of onset of mechanical activity.

TABLE III

Number and Length of Gap Junctions in Control and 3, 10, 20 and 33-mM TEA-Treated Canine Tracheal Smooth Muscle

	N[**]	No. GJ[††]	Distance (μm)[δ]	Junctions/1000 μm + SD	Length[Π] μm + SD
Control	10	160	49,785	3.36 ± 0.05	0.15 ± 0.05[†]
3 mM TEA[η]	10	278	54,597	5.35 ± 0.20[*]	
10 mM TEA[η]	10	311	66,251	5.04 ± 0.20[*]	
33 mM TEA Onset[ω]	5	146	28,349	5.10 ± 0.16[*]	
1 Hr[η]	10	248	49,925	5.69 ± 0.19[*]	0.17 ± 0.09[†]
20 mM TEA[η]	6	80	10,800	7.40 ± 0.40[*]	

[**] N = Number of tissues examined.

[††] Number of 5- or 7-layered junctions found in 3 to 5 grid squares (300 mesh) from each tissue scanned.

[δ] Distance = length of membrane (μm) of smooth muscle cells cut in transverse section measured from photographs of scanned grid squares.

[Π] Mean length (μm) of GJ measured from high magnification photographs (± SD).

[η] Tissues were exposed to TEA for 1 hour and fixed for thin section electron microscopy (EM).

[ω] Tissues were exposed to TEA and fixed for EM at a time when spontaneous mechanical activity was established (10 - 15 min).

[*] Indicates significant differences ($p < 0.01$) in the mean numbers between TEA-treated and control tissues.

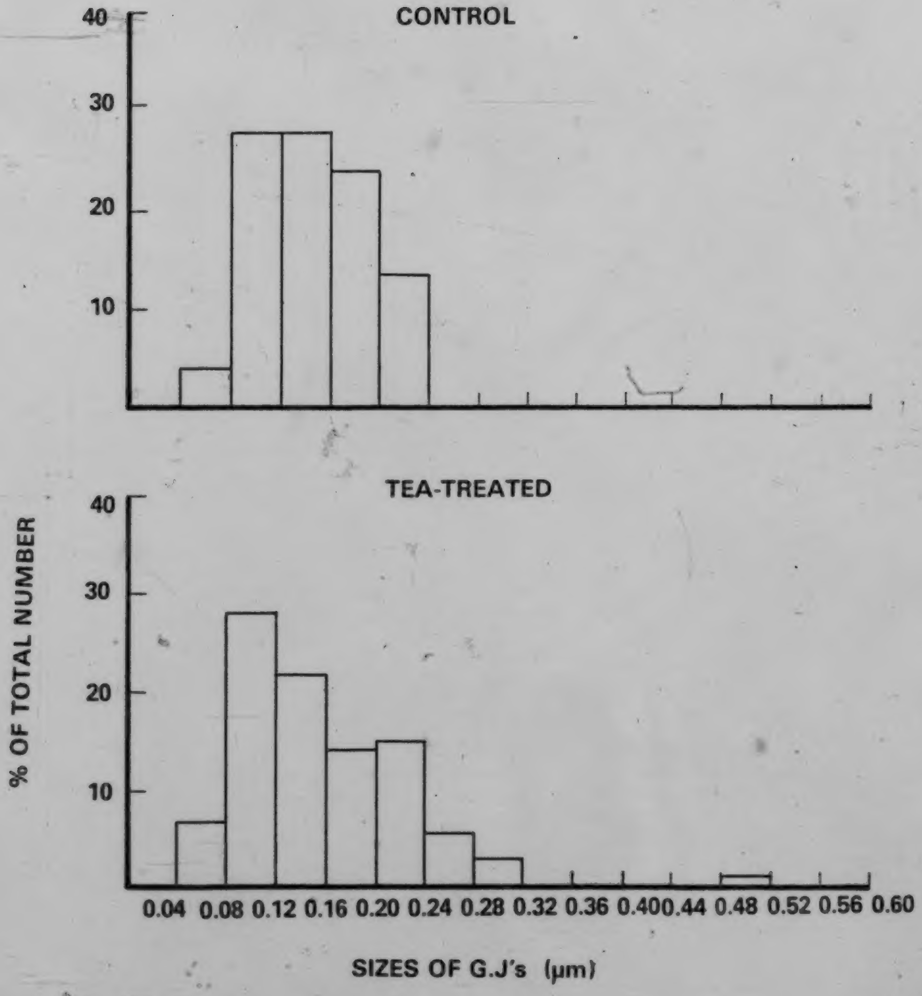
[†] Indicates no significant difference in the mean lengths of gap junctions between TEA-treated and control tissues.

Figure 14. Size distribution of gap junctions: the diameters were measured from high magnification micrographs of cells.

Control - diameters of 61 gap junctions are represented in the figure.

TEA-treated - diameters of 58 gap junctions are represented in the figure.

FIGURE 14



See Text For Details

Discussion

Gap junctions in the tracheal smooth muscle:

The claim that multiunit smooth muscles like the trachealis do not have gap junctions is contradicted by the results of this study. Gap junctions were regularly seen between smooth muscle cells of the canine trachealis (Figures 3 and 4). They could also be demonstrated by the freeze-fracture techniques (Chapter V).

Since our thin section EM studies were performed with tissues incubated *in vitro,* it could be argued that the gap junctions formed during this procedure. However, I have observed gap junctions between smooth muscle cells in tissues fixed *in situ* through a catheter in the left vetricle of the dog.

Dewey and Barr (1962) used permanganate as a fixative to demonstrate nexuses in smooth muscle. They claimed that glutaraldehyde fixative failed to preserve these structures. Use of permanganate as a fixative to demonstrate nexuses in smooth muscle has been questioned by many investigators. In a correlated thin-section EM and freeze-fracture study of different smooth muscles, Daniel et al (1976) have shown that glutaraldehyde fixation results in adequate preservation of these structures. Their study also showed that permanganate fixation results in formation of nexus-like structures in smooth muscle due to swelling of cells. Nexuses were described as 5-layered structures due to the fusion of the outer leaflets of the apposing cells by Dewey and Barr (1962). On the other hand, nexuses have been shown to be 7-layered structures, with a central gap about 2 nm wide using glutaraldehyde fixation and *en bloc* staining with uranyl acetate (Revel and Karnovsky, 1967 and Daniel *et al*, 1976).

Cable properties of tracheal smooth muscle:

Canine tracheal smooth muscle has been shown to exhibit cable properties as studied in vitro (Kroeger and Stephens, 1975 and Suzuki et al, 1976). The evidence for such comes from the electrophysiological studies of these authors. Using the partition-stimulation technique of Abe and Tomita (1968), these authors have shown that there is an exponential decay of the electrotonic potentials evoked by hyperpolarizing current pulses. They also reported a space constant of 1.6 mm for the canine trachealis which is many times the length of a smooth muscle cell. The space constant value increased to 2.8 mm after treatment with 33 mM TEA. Suzuki et al (1976) reported a space constant of 3.2 mm for the control tissues and a time constant of about 450 msec. These parameters were not measured after TEA treatment. However, these values are very high compared to values obtained in other smooth muscles.

Innervation of canine tracheal smooth muscle:

The studies reported here suggest that there is a cholinergic excitatory as well as an adrenergic inhibitory innervation in the canine tracheal smooth muscle. Field stimulation of the nerves resulted in a contraction which could be blocked by atropine. The relaxation seen after electrical stimulation of TEA-treated tissues could be blocked by propranolol suggesting a β-adrenergic mechanism. The absence of a non-adrenergic and non-cholinergic inhibitory innervation to this smooth muscle has been previously shown (Suzuki et al, 1976). These studies have also demonstrated the presence of cholinergic and adrenergic innervation by histochemical staining for acetylcholinesterase and catecholamines respectively. However, there seem to be species differences for such an inhibitory mechanism. In the tracheal smooth muscle of the guinea-pig, a non-adrenergic inhibitory innervation was described by Coburn and Tomita (1973), which has since been confirmed by others (Bando *et al*, 1973 and Coleman and Levy, 1974 and Richardson and Bouchard, 1975).

The nerve profiles in the canine trachealis contained three types of vesicles: small agranular vesicles, some small granular vesicles, and mostly large granular vesicles (Figures 4 to 6). The small agranular vesicles are generally considered to be present in cholinergic nerves (Burnstock, 1975). Studies also indicate that nerves containing noradrenaline have a preponderance of small granular vesicles (Burnstock, 1975). The large granular vesicles have been shown to take up and store catecholamines (Tranzer and Thoenen, 1968 and Furness *et al*, 1970), but are relatively resistant to depletion by reserpine (Taxi, 1965 and Bloom and Barrnett, 1966 and Clementi *et al*, 1966).

Effects of TEA:

The effects of TEA on the generation of conducted action potentials have been studied in a variety of smooth muscles where gap junctions were reported as absent and which had also been described as belonging to the multiunit type. Mekata (1971) reported stable membrane properties in the smooth muscle cells of the rabbit common carotid artery. The muscle exhibited cable properties as studied *in vitro* with a space constant of about 1.13 mm and a time constant of about 212 msec. The membrane had marked rectifying properties to depolarizing stimuli. Application of TEA (34.3 mM) in vitro, caused a depolarization of about 10 mV and action potentials were produced. The current-voltage relationship in TEA-containing solution revealed that the rectification was completely abolished. The membrane slope resistance, however, remained unchanged after TEA treatment.

Ito et al (1970) have studied the effects of TEA on the smooth muscle of the guinea pig stomach. The most striking change was an increase in the spike amplitude using a double-sucrose gap method. TEA caused an increase in the membrane slope resistance and abolished the rectifying property of the membrane.

In the bovine tracheal smooth muscle, which has stable membrane electrical properties, TEA caused depolarization and induction of spike activity (Kirkpatrick, 1975). The strong rectifying property of the membrane was also abolished by TEA. In the canine tracheal smooth muscle, which exhibits characteristics similar to that of bovine tracheal smooth muscle, TEA brought about similar changes. In addition, Kroeger et al (1975) reported an increase in the sapce constant on treatment with TEA, as described earlier. TEA also increased the transmembrane resistance and abolished the strong rectifying property to depolarizing stimuli.

Thus, the effects of TEA on vascular smooth muscle, guinea-pig stomach and the tracheal smooth muscles of the bovine and canine species were consistent with its known action, namely, blocking K^+ conductance leading to decreased rectification and in most cases an increase in transmembrane resistance.

TEA-induced structural effects:

The number of gap junctions is increased significantly in tissues exposed to TEA as compared to the number in control tissues (Table III). The resultant increase in the gap junctional membrane area (from 0.05% in control to 0.09% in the TEA-treated tissues) could serve to decrease the junctional resistance for current flow between the coupled smooth muscle cells.[1] The observed increase in the space constant of the TEA-treated tissues could thus be a consequence of either an increase in the transmembrane resistance or a decrease in the junctional resistance or both. Implicit here are the assumptions that gap junctions act as low-resistance pathways for current flow between smooth muscle cells and the space constant is determined solely by values of membrane and junctional resistances.

Gap junctions as pathways for current flow in smooth muscle:

In smooth muscle, the evidence supporting gap junctions as low-resistance pathways for current flow between cells is only circumstantial, based on the proximity of the apposing membranes (a gap of 2 nm separates the outer membrane leaflets) and on the permeability and coupling properties of similar contacts between epithelial cells. There have been no direct measurements of the resistance of these junctions. Indirect estimates of the specific resistance of the nexus at the intercalated disc of canine myocardium (Spira, 1971), a structure resembling the gap junction of smooth muscle, are however consistent with their presumptive role as low-resistance contacts. There is no general correlation between the presence of electrical coupling and the presence of gap junctions in smooth muscle. Daniel et al (1976) in their studies on the longitudinal smooth muscle layer of the dog intestine could not demonstrate gap junctions by both thin-section EM and freeze-fracture techniques. There is overwhelming evidence for electrical coupling between the cells in this layer. On the other hand, gap junctions could be demonstrated in the main circular layer of dog intestine by the same fixation techniques as well as by freeze-fracturing. Recently, in rat myometrium studied in situ (Garfield et al, 1977) it has been shown that large gap junctions can form rapidly under physiological control by hormones, but even prior to the appearance of gap junctions the tissues showed evidence of good electrical coupling as studied in vitro (Kuriyama et al, 1976 and Lodge and Daniel, 1973).

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[1] The junctional membrane area was calculated by multiplying the mean measured diameter of gap junctions by their mean number per 100 μm length of membrane.

The presence of gap junctions in the canine tracheal smooth muscle may provide one basis for coupling and their rapid formation after TEA treatment could account for a decreased junctional resistance. There is a parallelism between the functional changes and the structural effects induced by TEA in this smooth muscle suggesting that gap junctions could be involved in the changed properties.

The mechanism whereby TEA can induce rapid formation of gap junctions in canine tracheal smooth muscle is either through *de novo* synthesis of membrane proteins involved in their assembly or through aggregation of pre-existing membrane proteins by accretion to structures recognized as gap junctions in thin sections. These possibilities are considered in Chapters IV and V.

The changes in the biophysical properties of canine trachealis upon TEA treatment could result from its effect on blocking K^+ conductance; increasing the transmembrane resistance; abolishing the rectification and/or from its ability to increase the number of gap junctions leading possibly to a decreased junctional resistance. The ability of a simple chemical agent to increase the number of gap junctions rapidly and reproducibly suggests its use as a tool to provide more direct evidence about the role of gap junctions in cell-to-cell coupling of smooth muscles and possibly in other tissues.

CHAPTER III

EFFECTS OF 4-AMINOPYRIDINE ON CANINE TRACHEAL SMOOTH MUSCLE

CHAPTER III

Introduction

TEA induces spontaneous electrical and mechanical activities in the canine tracheal smooth muscle in vitro (Chapter II). The effects of TEA were interpreted as being consistent with its known action namely, blocking K conductance in smooth muscle (see Chapter II). From the results of the structural studies reported in Chapter II, it was concluded that the functional changes brought about TEA in tracheal smooth muscle could arise not only from its effect on blocking potassium conductance but also from its ability to induce formation of gap junctions.

The compound 4-Aminopyridine (4-AP) has been shown to block selectively potassium channels of cockroach giant axons, squid axons and a variety of other excitable membranes (see Discussion). Although the selectivity of action of 4-AP has been established, its site and mode of action resulting in potassium conductance blockade has been shown to differ from those of TEA. In the light of such differences, I wanted to test if 4-AP was also capable of bringing about a conversion from the multiunit to the single-unit state of canine tracheal smooth muscle in vitro with the attendant structural changes. The effects of 4-AP on smooth muscle have not been investigated to date.

The objectives of the present investigation were:

i) To test if 4-AP was capable of inducing phasic mechanical activity in tracheal smooth muscle in vitro;

ii) If so, to establish the dose-response relationship and the time-course of action;

iii) To test effects of electrical stimulation in the presence of 4-AP;

iv) To test if this compound was capable of inducing formation of gap junctions;

v) To test if the structural changes can be prevented by blocking the mechanical effects induced by 4-AP.

Materials and Methods

Tissue preparation: Tracheal smooth muscle strips were obtained from dogs using the methods described in Chapter II and mounted in a 20 ml organ-bath at 37° C to record isometric tension. An initial one gm tension was applied to all tissue strips.

Dose-response relationship to 4-AP: Three concentrations of 4-AP were used in these studies, 1, 3 and 10 mM. After incubating for 1 hour the tissues were fixed for electron microscopy (see Chapter II).

Time-course of 4-AP effect: Two concentrations were chosen, 1 and 10 mM. Tissues were fixed for electron microscopy at a time when phasic mechanical activity was established (10 to 15 minutes) and one hour after addition of the drug.

Solutions: 4-AP was dissolved in water and the pH of this solution was adjusted to 7.4 with 0.1 N HCl to give a final concentration of 0.1 M. This stock solution was diluted with Krebs solution to achieve the desired bath concentrations of 1, 3 and 10 mM. The Krebs solutions containing the different 4-AP concentrations were prepared by replacing an equimolar amount of NaCl with the drug.

Electrical stimulation: Field stimulation was achieved through a pair of platinum electrodes placed concentrically around the tissue strips, attached to a Grass Stimulator, Model S9. Both nerve and direct muscle stimulations were obtained by varying the stimulus parameters (see Methods in Chapter II).

Electron microscopy: Tissues were fixed after the necessary incubation period and processed for electron microscopy as per Methods described in Chapter II.

Quantitation of gap junctions: The gap junctions between smooth muscle cells in thin sections of tissues cut in transverse orientation were quantitated as already described in Methods, Chapter II.

Results

Effects of 4-AP on isometric tension in the canine trachealis:

Tissues incubated in Krebs solution failed to show any mechanical response (Chapter II). On adding solution containing 4-AP, the tension rose almost immediately. There was no indication of a lag period between addition of the drug and the onset of mechanical response (to concentrations 1, 3 and 10 mM 4-AP). This was in contrast to the effects seen with TEA. The tension reached a steady value and the tissue started to exhibit spontaneous rhythmic contractions. The tone fluctuated, but it did not reach the original base-line (Figure 15). Upon addition of Krebs solution, the tension fell sharply to the base-line. Some tissue strips which were exposed to 10 mM 4-AP had to be washed repeatedly with Krebs solution to restore base-line tension. Tissues from seven animals were studied.

The maximum active tension attained after addition of 4-AP was dose-related, being higher for larger doses. Phasic activity was seen consistently with 3 and 10 mM 4-AP. With 1 mM 4-AP, phasic activity was not discernible on the trace in some strips. The response, when present, consisted of a steadily increasing tone which reached a steady-state and remained unchanged thereafter.

Table IV shows the maximum active tension attained after exposing the tissues to the three 4-AP concentrations. The maximum tension attained with 10 mM 4-AP was taken as 100%. The results from eleven experiments (33 strips) are included in this study.

Electrical stimulation of tissues:

The effects of electrical field stimulation on the mechanical response induced by 1, 3 and 10 mM 4-AP were studied in tissues from six animals. The mechanical effects are illustrated in Figure 16. When the isometric tension reached a steady-state level after

TABLE IV

Maximum Active Tension in 4-AP-Treated Tissues[+]

n[++]	1 mM	3 mM	10 mM
33	38.8[*] (3.35)	55[*] (3.0)	100%

[+] The maximum active tension attained in tissues treated with 10 mM 4-AP for one hour was taken as 100% response.

[++] Number of tissue-strips from 11 animals.

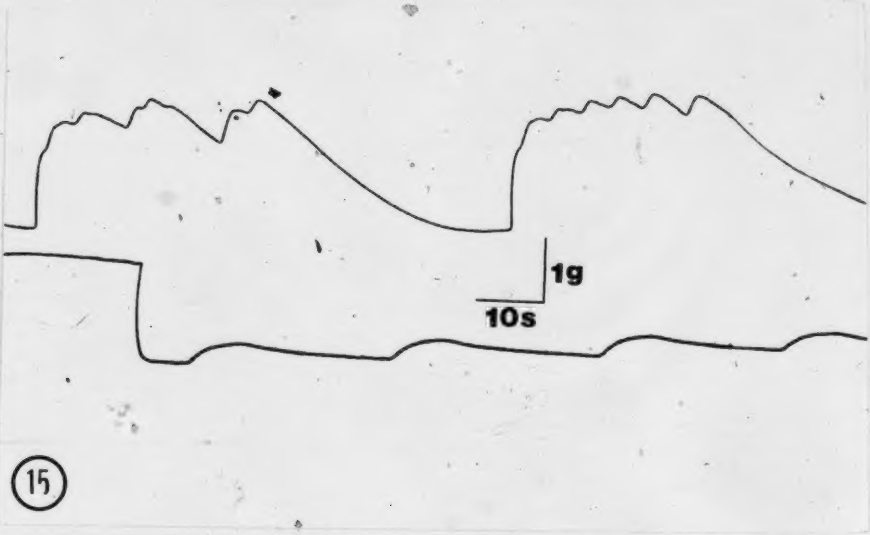
[*] Values are significantly lower ($p < 0.001$) than maximum response.

Maximum response to 1 mM 4-AP are significantly lower ($p < 0.01$) than response to 3 mM 4-AP.

Values in parentheses are ± S.E.

Figure 15. Fluctuation of tension with phasic mechanical activity of canine tracheal smooth muscle strip treated with 10 mM 4-AP.

Figure 16. Electrical field stimulation of trachealis strips after treatment with 1, 3 and 10 mM 4-AP. Stimulus parameters are: 15 V, 5 msec. and 60 Hz. Arrows indicate stimulus duration. Consult text for the nature of the mechanical responses.



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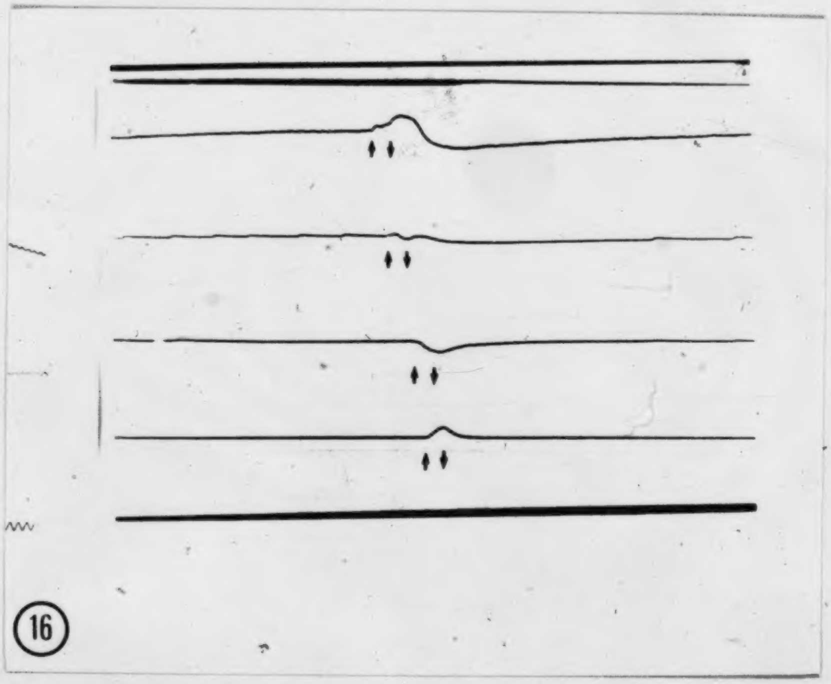

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application of 4-AP, the tissues were stimulated electrically. After treatment with 1 mM 4-AP, field stimulation of either nerves or direct muscle stimulation resulted in an increase in tone, during the period the stimulus was applied. The tension returned to the pre-stimulus level when the stimulus was discontinued. After treatment with 3 and 10 mM 4-AP, field stimulation caused a relaxation during the stimulus duration. On turning the stimulus off, the tension was restored to the pre-stimulus level. The relaxation seen after 10 mM 4-AP was larger in amplitude than that seen after 3 mM 4-AP treatment. The maximum active tension, as shown above, was also higher with 10 mM 4-AP than with 3 mM 4-AP. This suggests that the magnitude of relaxation after field stimulation is related to the basal tone in the preparation.

This relaxation was blocked by propranolol (10^{-6} M) suggesting that as with TEA a β-adrenergic response to released amines. The failure to elicit a contractile response to field stimulation after 3 and 10 mM 4-AP could be due to the muscle response being at near maximal level. This was true in tissues exposed to higher concentrations of TEA (Chapter II, Results section).

Effects of atropine on 4-AP-induced mechanical response:

The initiation of phasic mechanical activity in the canine trachealis by TEA is not blocked by atropine suggesting that the effect is not dependent on the release of acetylcholine from the intrinsic nerve endings (Chapter II, Results). The phasic activity induced by 4-AP could be the result of either release of transmitter from the nerves or a direct effect on the smooth muscle cell membrane to block potassium conductance or both. In order to distinguish between these possibilities, atropine, a muscarinic cholinergic blocking agent, was used.

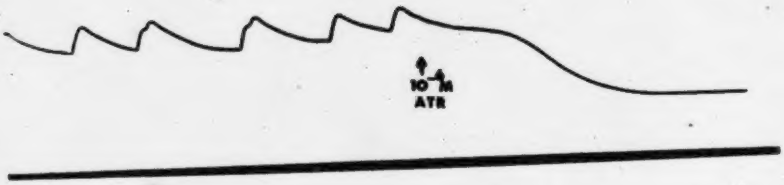
Tissues were exposed to 4-AP (1, 3 and 10 mM) and when phasic mechanical activity was established, atropine was added to the bath (to reach a final concentration of 10^{-7} to 10^{-6} M). The phasic as well as the tonic responses were completely abolished by atropine (Figure 17) (n = 4). Tissues were also preincubated with the same concentrations of atropine for 10 minutes and later exposed to the three concentrations of 4-AP. Mechanical activity was not seen in any of these tissues (n = 4).

After atropine treatment, field stimulation of nerves failed to elicit any response. When the duration of the pulse was increased (from 5 msec up to 50 msec), field stimulation caused a contraction during the stimulus duration. The tension reached the base-line on turning the stimulus off. Phasic mechanical activity could not be initiated in these tissues by electrical field stimulation.

These findings suggest that the 4-AP-induced mechanical effects are due to release of acetylcholine from the nerve endings. If so, external application of acetylcholine must elicit a phasic mechanical response, simulating the actions of 4-AP. However, application of Ach (10^{-8} to 10^{-7} gm/ml) caused only a sustained increase in the tone of the tissue, with no evidence of a phasic component. It is difficult to explain the initiation of phasic activity solely by release of transmitter from nerves. The tone in the tissues after 4-AP treatment could be due to the released transmitter. Raising the tone

Figure 17. Mechanical response of canine tracheal smooth muscle strip after exposure to 10 mM 4-AP. Arrow indicates addition of 10^{-6} M atropine.

Consult text for the nature of the mechanical response.



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with field stimulation of muscle in atropine-treated tissues also failed to initiate phasic activity. This tends to rule out the dependence of phasic response on tone.

Gap junctions in 4-AP-treated tissues:

Dose-response relationship on structure:

The effect of treatment in vitro for one hour with two different concentrations of 4-AP (1 and 10 mM) on the number of gap junctions in the canine tracheal smooth muscle was studied using electron microscopy. The number of gap junctions seen as 5- or 7-layered structures with a central 2 nm gap in thin sections of tissues cut in cross-sections was counted. The number of gap junctions in control tissues was 3.82 ± 0.12 per 1000 μm length of membrane. The number of gap junctions in tissues treated with 1 mM 4-AP was 8.11 ± 0.33 per 1000 μm membrane length. The number of gap junctions in 10 mM 4-AP-treated tissues was 10.80 ± 0.45 per 1000 μm membrane length. The latter two values were significantly higher ($p < 0.01$) than the value in control tissues (Table V) but not from one another. As in TEA-treated tissues, the gap junctions were typically found between processes connecting two smooth muscle cells.

Time-course of 4-AP effect on structure:

Two concentrations of 4-AP were chosen for this study, namely, 1 and 10 mM. Tissues were fixed after exposure to 4-AP at a time when

TABLE V

Number of Gap Junctions in Control and 1 and 10 mM 4-AP-Treated Canine Tracheal Smooth Muscle[†]

	n	No. GJ	Distance (μm)	GJs/1000 μm ± SD
Control	7	110	28,944	3.82 ± 0.12
1 mM 4-AP				
Onset	3	136	17,643	7.71 ± 0.25*
1 Hour	7	210	25,911	8.11 ± 0.33*
10 mM 4-AP				
Onset	3	114	11,801	9.72 ± 0.30*
1 Hour	6	315	29,032	10.80 ± 0.45*

[†] Results and experimental design as in Table III.

*Values are significantly higher ($p < 0.05$) than values in control tissues.

spontaneous mechanical activity was established (10 to 15 minutes after addition of the drug) as well as one hour after addition of the drug. The number of gap junctions in tissues fixed at 10 - 15 minute and one hour after exposure to 4-AP (1 mM) respectively was: 7.71 ± 0.45 per 1000 μm membrane length and 8.11 ± 0.33 per 1000 μm membrane length. The number of gap junctions in tissues fixed at 10 - 15 minute and one hour after exposure to 10 mM 4-AP respectively was 9.72 ± 0.3 per 1000 μm membrane length and 10.80 ± .045 per 1000 μm membrane length (Table V). These values were significantly higher ($p < 0.01$) than the values in control tissues. There was no significant difference between values in 10 - 15 minute and one hour treated tissues ($p > 0.05$).

Effects of atropine on 4-AP-induced formation of gap junctions:

Tissues were treated with 10^{-7} M atropine for 15 minutes and later exposed to either 1 mM or 10 mM 4-AP for one hour and processed for electron microscopy for determining the number of gap junctions. The mean number of gap junctions in control tissues was 4.32 per 1000 μm length of membrane and in tissues treated only with atropine was 3.3 per 1000 μm length of membrane. Tissues treated with 1 mM and 10 mM 4-AP for one hour had respectively 8.35 and 15.68 per 1000 μm membrane length. These latter values were significantly higher ($p < 0.01$) than the number in control or atropine-treated tissues. Tissues pretreated with atropine for 15 minutes and later exposed to 1 mM or 10 mM 4-AP also had significantly greater number ($p < 0.01$) than in control or atropine-treated tissues (8.08 per 1000 μm length of membrane in 1 and 10 mM 4-AP tissues respectively). These latter values, however, were slightly less than in the corresponding non-atropine-treated tissues (Table VI), but not significantly different ($p > 0.05$).

Effects of acetylcholine on gap junction formation:

The number of gap junctions in tissues from four animals treated with acetylcholine (10^{-7} M) for one hour was determined. The mean number in control tissues was 4.20 per 1000 μm and in Ach-treated tissues was 3.50 per 1000 μm membrane length. The latter value was not significantly different ($p > 0.05$) from the control value.

Figure 18 shows an electron micrograph of smooth muscle cells cut in transverse orientation in a thin section of 4-AP-treated tissue fixed one hour after addition of the drug. The smooth muscle cells have very irregular shapes and two gap junctions can be seen at points indicated. The surface membrane as well as the cytoplasmic organelles are preserved well. In Figure 19, the Golgi apparatus can be seen to have a swollen appearance in two of the cells. This swelling of Golgi cisternae is a consistent feature in tissues treated with 4-AP. No such changes were seen with TEA. Some smooth muscle cells show vacuolation after 4-AP treatment. This was evident in some of the nerve bundles in such tissues. Figure 20 shows one such nerve bundle showing vacuolation of the Schwann cell cytoplasm.

These results suggest that rapid formation of gap junctions still occurs when the mechanical activity induced by 4-AP is blocked by atropine. Treatment with Ach does not result in an increase in the number of gap junctions. Thus, the ability of 4-AP to induce gap junction formation in the canine tracheal smooth muscle is not dependent on a

TABLE VI

Effect of Atropine on the Number of Gap Junctions in 4-AP-Treated Canine Tracheal Smooth Muscle

(Number of Gap Junctions/1000 μm Membrane Length)

Expt. No.	Control	Control + Atr.	1 mM 4-AP	1 mM 4-AP + Atr.	10 mM 4-AP	10 mM 4-AP + Atr.
1	4.1	3.9	6.9	4.6	23.0	7.5
2	6.4	5.2	10.9	8.5	7.5	6.2
3	3.8	2.9	10.9	7.6	19.3	3.8
4	3.0	1.2	10.2	11.5	12.9	6.0
X	4.32	3.30	8.35*	8.08*	15.68*	5.88*

* Values are significantly higher than ($p < 0.05$) values in control and control + atropine-treated tissues. The significance of the differences in the mean values of individual experiments was analyzed using an unpaired t Test.

Consult the text for the experimental design.

Results are analyzed as per methods described in Table III.

Figure 18. Smooth muscle cells from canine trachealis in cross-section. The field shows cells from tissues fixed after treatment with 10 mM 4-AP for one hour. Two gap junctions (arrows) can be seen. Magnification x 30,000.

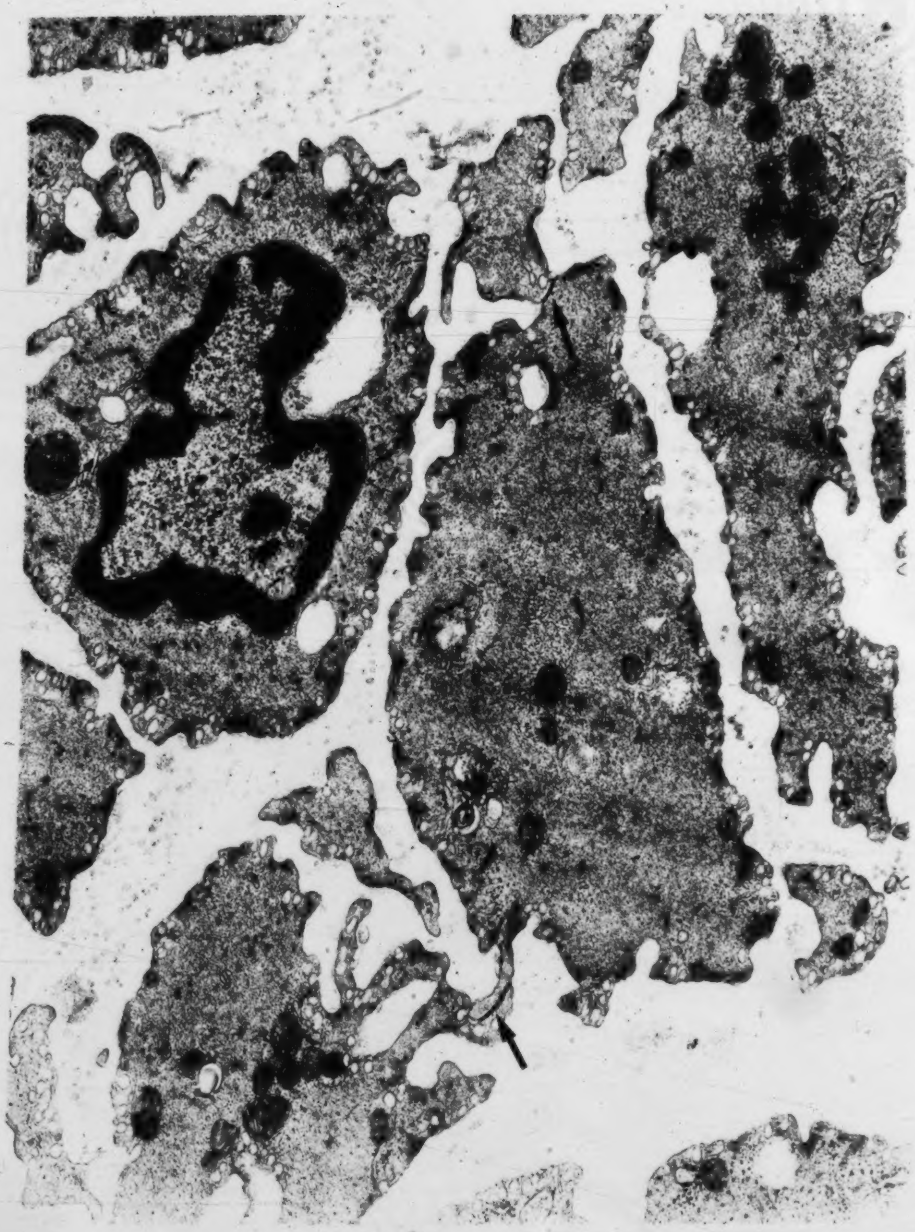

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Figure 19. Smooth muscle cells from tissues fixed after treatment with 10 mM 4-AP for one hour. The Golgi cisternae (G) in two cells are swollen with myelin figures. Arrow-heads - gap junctions. Magnification x 30,000.

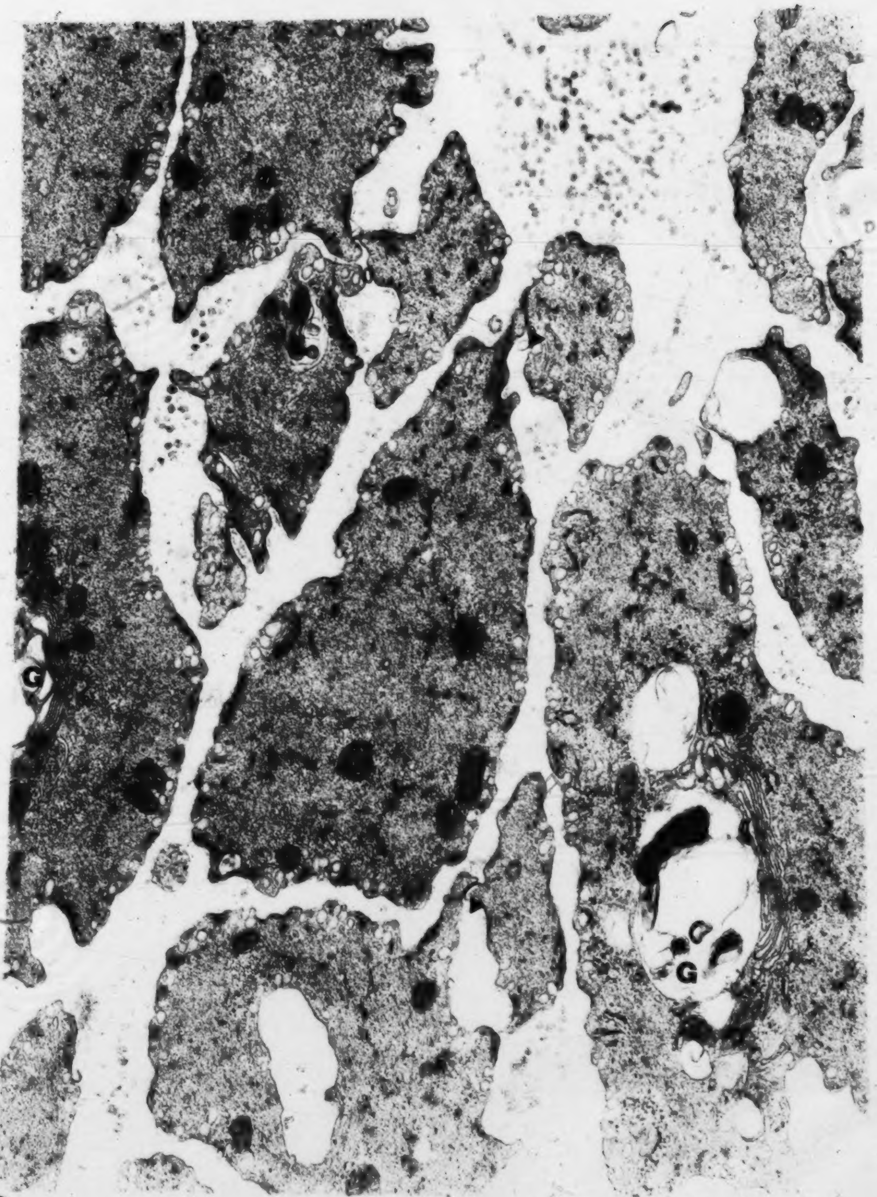

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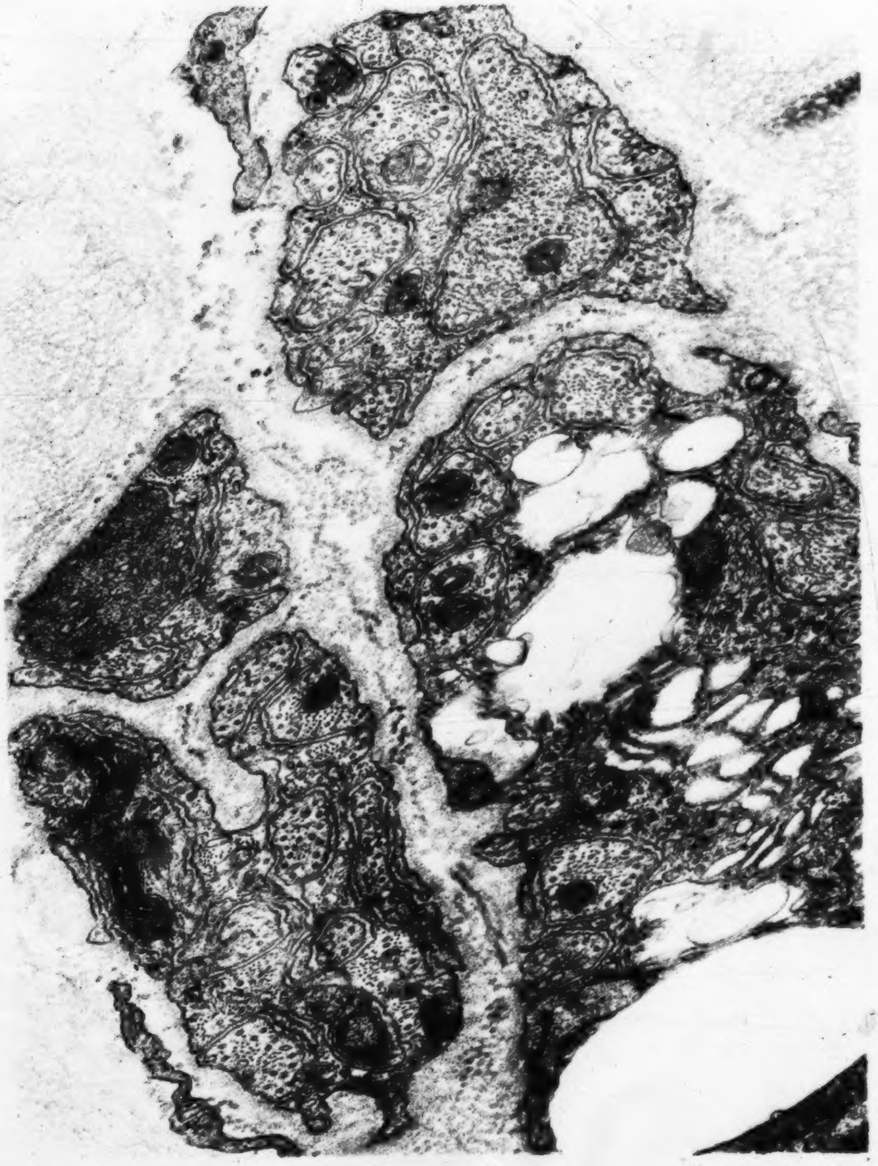
Figure 20. Nerve fibres in canine trachealis muscle fixed after treatment with 10 mM 4-AP for one hour. Extensive vacuolation of the Schwann cell cytoplasm is evident. Magnification x 38,000.



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cholinergic component of action.

Discussion

The compound 4-AP acts as a convulsant in the nervous systems of vertebrates (Le Meignan et al, 1969) and invertebrates (Pelhate et al, 1972). On the single axons of the cockroach, 4-AP has been shown to prolong the falling phase of the spike and to reduce the delayed rectification (Pelhate and Pichon, 1974). On external perfusion of the giant axon of cockroach, 4-AP reduces the delayed (potassium) current under voltage-clamp conditions. This reduction of potassium current was found to be reversible and independent of the membrane potential. Experiments with high K solutions indicate that 4-AP is effective regardless of the direction of K current flow. On the basis of these effects it was concluded that 4-AP was a very selective blocker of potassium conductance in cockroach axons and was shown to be about 2000 times more potent than TEA.

The selectivity of block of potassium channels by 4-AP has been tested in studies on the axons of squid (Meves and Pichon, 1975 and Yeh et al, 1976 a and b) and Myxicola (Schauf et al, 1976) and the frog node of Ranvier (Wagner and Ulbricht, 1975). In the giant axon of the squid (Yeh et al, 1976 a and b) have shown that, in addition to reducing potassium currents, 4-AP and its analogues exhibit voltage-, time- and frequency-dependent characteristics. Similar effects were observed in the frog node of Ranvier (Wagner and Ulbricht, 1975).

In the squid axon, TEA is also known to reduce the current through potassium channels suggesting an overt similarity to the action of 4-AP. There are, however, several differences in the mechanisms of action of 4-AP and TEA in the squid giant axon as exemplified by the following observations:

i) TEA is effective only when internally perfused. On the other hand 4-AP is effective either from outside or from the inside of the membrane;

ii) TEA has no effect on the membrane potential, but prolongs the action potential duration considerably. Aminopyridines, on the other hand, depolarize the membrane giving rise to repetitive firing of action potentials, but their effect on the duration of action potential is minimal;

iii) When the membrane is depolarized to a greater extent under voltage-clamp conditions, the block induced by aminopyridines is relieved. A similar effect is seen when the pulse duration is increased. The TEA-induced block under these conditions is accentuated. According to the kinetic model proposed by Yeh et al (1976 a and b), aminopyridine molecules are assumed to interact with K channels by binding to sites in the channel. At larger depolarizations, when the K channels remain open, result in the release of aminopyridine molecules from the channels. This results in removal of 4-AP block of K current. TEA, on the other hand, is assumed to bind when the channels remain open and such depolarizations tend to increase the block of K channels.

iv) Aminopyridines are effective in reducing both the inward and the outward K currents as has been shown for the cockroach giant axon, while TEA can only block the outward K current.

v) For the TEA-induced block to take place, the K channels are required to be open (Armstrong, 1966). This results in relief from 4-AP-induced block.

The effect of 4-AP on adrenergic transmission in the vas deferens of the rabbit has been studied by Johns et al (1976). It was shown that this compound potentiated the responses to transmural stimulation. Although the precise mechanism was not determined, it seemed that the effect might be due to an increased transmitter release, possibly by prolonging the duration of the nerve action potentials. 4-AP was also shown to be more potent than TEA in this effect.

In the canine tracheal smooth muscle, 4-AP induced phasic mechanical activity. The onset of action was almost immediate. The response consisted of a steadily increasing tone which reached a plateau whereupon the tissue started to exhibit rhythmic contractions. As shown in the Results, the maximum active tone was dependent on the dose of the drug employed (Table IV). Atropine (10^{-7} M) blocked the tonic as well as the phasic components of the response. The atropine-treated tissue was not capable of phasic activity even when the tone was raised by electrical stimulation using long duration pulses.

The results with exposure of the tissues to Ach revealed that the activity consisted of a sustained increase in tone. Stephens and Kroeger (1970) have shown that application of Ach (10^{-8} to 10^{-4} M) caused dose-dependent tonic contractions and a myogenic response to quick stretch could not be elicited. The tonic phase of the mechanical response after application of 4-AP could be due to release of Ach from the intrinsic nerve-endings, but the basis of the phasic component on such a mechanism is difficult to explain. It will be of interest to study the ability of 4-AP to cause a myogenic response in tracheal smooth muscle. Since myogenic response can only be initiated in tissues exposed to agents which result in blockade of K conductance, this will serve to confirm the mechanism of action of 4-AP. Electrophysiological studies are also needed to resolve this question.

The possibility remains that, in addition to releasing excitatory transmitter from nerves, 4-AP also has a direct smooth muscle effect resulting in blocking K conductance. The number of gap junctions in tissues exposed to atropine followed by 4-AP was still higher than in untreated control tissues, but lower than in the drug-treated tissues. This slight reduction could have been due to elimination of a cholinergic component of the response or absence of mechanical activity. However, treatment of tissues with acetylcholine did not result in increased gap junction formation. Thus, an involvement of a cholinergic component of 4-AP effect on gap junction formation seems to be unlikely in this tissue.

Comparison of 4-AP and TEA effects on the canine trachealis:

i) Both these compounds are capable of inducing phasic mechanical activity in this smooth muscle. With 4-AP, the effect was rapid in onset, but with TEA there was a lag period after addition of the drug.

ii) The effects of electrical field stimulation on the 4-AP-induced mechanical response was also similar to those seen with TEA.

iii) Atropine blocks both the tonic and the phasic components of the mechanical activity induced by 4-AP whereas it has no effect on the TEA-induced phasic activity. Atropine does diminish the tone after TEA treatment, but the tone recovers with time.

iv) The ability to induce increased formation of gap junctions is shared by both compounds. The effects are rapid in that increased gap junction formation takes place within 10 to 15 minutes after exposure of the tissues to the drugs. The number of gap junctions in 4-AP-treated tissues is consistently higher than in tissues treated with TEA. Atropine does not block gap junction formation by 4-AP; gap junction formation is not sufficient to induce phasic activity.

v) Tissues treated with 4-AP consistently showed swelling of the Golgi cisternae as well as vacuolation of the cytoplasm. Such changes were also seen in the nerve bundles in the tissues. Such changes were not seen in tissues treated with TEA.

CHAPTER IV

EFFECTS OF CYCLOHEXIMIDE ON TEA- AND 4-AP-INDUCED FORMATION OF GAP JUNCTIONS

CHAPTER IV

Introduction

From the results of the studies reported in the previous chapters, it is evident that potassium conductance blockers like TEA and 4-AP induce spontaneous phasic mechanical activity in vitro in the canine tracheal smooth muscle. The structural studies revealed that the number of gap junctions in tissues exposed to TEA and 4-AP was increased significantly as compared to untreated control tissues and this increase was evident as early as 10 minutes after addition of the drugs. When the phasic mechanical activity induced by 4-AP was abolished by atropine pretreatment, the number of gap junctions appeared to diminish, suggesting a relationship between acetylcholine release, induction of spontaneity and formation of gap junctions. However, factors other than acetylcholine release must also have been involved since atropine did not reduce the number of gap junctions to control levels and atropine had no effect on either the spontaneous activity or the increase in gap junctions by TEA treatment. Also treatment of tissues with acetylcholine did not result in an increase in the number of gap junctions either.

In vitro formation of gap junctions has been studied in a variety of excitable as well as non-excitable cell systems (see Discussion). In some of these experiments inhibition of protein synthesis did not inhibit gap junction formation. Thus in some cells de novo protein synthesis is not necessary for gap junction formation, while in others it is required for junction formation.

Since TEA and 4-AP consistently and rapidly increased the number of gap junctions in vitro in canine trachealis, studies were undertaken to investigate the dependence on protein synthesis of this outcome. Cycloheximide (CHX), a protein synthesis inhibitor, was used in these studies. Incorporation of radioactive leucine into TCA-insoluble fraction was measured as an index of de novo protein synthesis in canine trachealis and the ability of different concentrations of CHX to inhibit it.

Materials and Methods

Tissue preparation:

Strips of canine tracheal smooth muscle tissues were obtained as per methods described in Chapter II. The tissues were allowed to recover in Krebs solution at 37° C for one hour before the experiments.

^{3}H-Leucine incorporation studies:

After the recovery period, the tissue strips were incubated in Krebs solution at 37° C containing 0.25 µmoles of ^{3}H-leucine of specific activity 50 Ci/mmole for one hour. The solution was bubbled with a mixture of 95% O_2 - 5% CO_2. At the end of the incubation period, an equal volume of Krebs solution containing 5 mM leucine (final leucine concentration 2.5 mM) was added. The tissues were homogenized by a Polytron tissue homogenizer (20 second cycles x 4) and the homogenate spun at 4000 x g in a Sorvall centrifuge. The supernatant was taken as the starting material. Protein content of the supernatant was estimated by the Lowry et al (1951) method.

An equal volume of 10% TCA was added to the supernatant and spun in a table-top centrifuge for 20 minutes. The precipitate was washed twice with TCA and digested with 0.5 ml of 0.4 N KOH at 65° C for 5 minutes. To this digest, 3 ml of 10% TCA was added and centrifuged for 20 minutes. The pellet was washed once with 10% TCA and spun. The resulting pellet was washed successively with 1:1 mixture of ethanol:ether, followed by ether and dissolved in NCS tissue solubilizer. A 0.2 ml aliquot of the NCS-dissolved pellet was plated in a scintillation vial to which 10 ml of Bray's solution was added and counted in a Beckman Scintillation counter

Effect of CHX on ^{3}H-leucine incorporation:

The tissue strips were preincubated in Krebs solution at 37° C containing 1 or 5 mM CHX for 30 minutes before addition of ^{3}H-leucine and processed for counting of radioactivity as described above.

Expression of results:

Results were expressed as cpm/mg protein in the 4000 x g supernatant.

Mechanical studies:

Isometric tension was monitored in tissues mounted vertically in a

20-ml organ-bath by attaching one end to a Grass-FT03 force transducer as described in Chapter II. Six tissue strips were used in each experiment: and were treated as follows: 1) control strip incubated in Krebs solution for one hour; 2) strip incubated in CHX (5 mM) for 90 minutes; 3) strip exposed to 20 mM TEA for one hour; 4) strip preincubated for 30 minutes with 5 mM CHX and later exposed to 20 mM TEA-containing solution with 5 mM CHX for one hour; 5) a strip incubated with 10 mM 4-AP for one hour; and 6) a strip preincubated for 30 minutes with 5 mM CHX and later exposed to 10 mM 4-AP solution containing 5 mM CHX for one hour.

Electron microscopy:

At the end of the incubation period, the muscle strips were fixed for electron microscopy as described in Chapter II.

Quantitation of gap junctions:

The number of gap junctions in thin sections of tissues cut in cross-section was determined as mentioned in Chapter II.

Results

A. ^{3}H-Leucine incorporation into TCA-insoluble fraction of canine tracheal smooth muscle:

Incorporation of ^{3}H-leucine into TCA-insoluble fraction was measured. Table VII summarizes the extent of incorporation in tissues from five dogs and the effects of CHX (1 and 5 mM) treatment. Tissues preincubated with 1 mM CHX for 30 minutes before addition of radioactive leucine showed only a 35 - 52% inhibition of protein synthesis. In later experiments, a concentration of 5 mM CHX was used. This dose resulted in about 95% inhibition of ^{3}H-leucine incorporation. In studies dealing with the effect of inhibition of de novo synthesis of protein on TEA and 4-AP-induced formation of gap junctions, a concentration of 5 mM CHX was used.

B. Mechanical effects:

Isometric tension was recorded in tissues from six animals. Incubation of tracheal smooth muscle strips in either Krebs solution or solution containing 5 mM CHX did not elicit any mechanical activity during the incubation period of 90 minutes (Chapters II and III).

Tissues treated with 30 mM TEA or 10 mM 4-AP after a 30 minute preincubation in Krebs solution showed phasic mechanical activity (Chapters II and III).

Tissue strips from three animals preincubated with 5 mM CHX-containing Krebs solution for 30 minutes and later exposed to either 20 mM TEA or 10 mM 4-AP for one hour showed spontaneous rhythmic mechanical activity. However, tissue strips from three other animals failed to show any mechanical response when treated in the same manner.

TABLE VII

Effects of Cycloheximide (CHX) on ^{3}H-Leucine Incorporation into the TCA-Insoluble Fraction of Canine Trachealis*

	Control	1 mM CHX	5 mM CHX
cpm/mg protein	12,452	6,000	700
"	14,113	8,200	1,150
"	20,809	10,100	1,250
"	17,231	7,500	550
"	6,500	2,570	145
% incorporation of control		47.5 ± 6.9	5.0 ± 2.3

*Consult text for experimental design.

Results from five experiments are given.

C. Effects of CHX on structure:

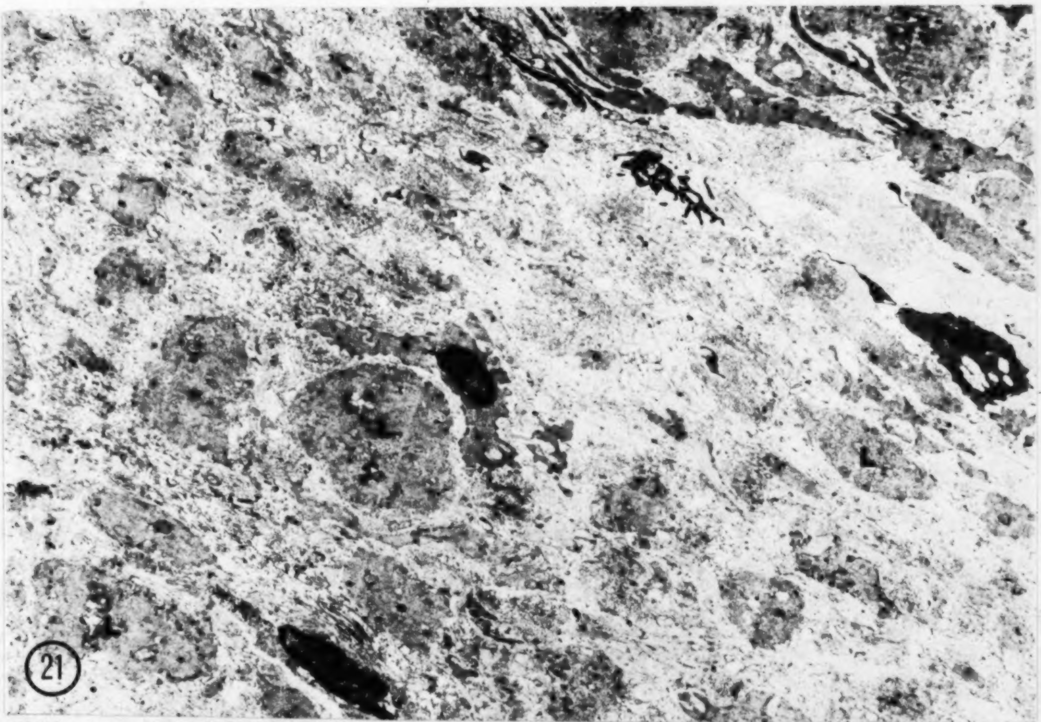
i) General appearance: Treatment with CHX resulted in structural damage to smooth muscle cells. Figures 21 and 22 show a group of smooth muscle cells from tissues fixed after treatment with 5 mM CHX. Figures 23 and 24 show smooth muscle cells from tissues treated with 5 mM CHX and either 20 mM TEA (Figure 23) or 10 mM 4-AP (Figure 24) from the same animal. The damaged cells were stained less than normal cells. The surface caveolae were few in number and in many cells were absent. The surface membrane continuity was lost in many of the damaged cells. There were many cytoplasmic vacuoles and the myofilaments were disrupted. Many of the mitochondria observed were severely damaged and those seen were in orthodox configuration. Gap junctions were not seen either between the damaged cells or between one of these and the neighbouring healthy smooth muscle cells. The damaged cells had a swollen appearance. They were typical "light" cells as recently described in the rat myometrium seen after metabolic inhibition or mechanical damage (Garfield and Daniel, 1976).

The extent of such damage, as judged by the proportion of damaged cells, was determined in tissues which showed a mechanical response to TEA or 4-AP after preincubation with 5 mM CHX as well as in those which failed to show any mechanical activity. Table VIII summarizes the quantitative aspect of damage induced by CHX treatment in the tracheal smooth muscle.

The damaged cells were not traced for measurement of the membrane length when estimating the number of gap junctions per unit length of membrane (see ii) and iii) below).

Figure 21. Low-magnification view of smooth muscle cells from tissues fixed after exposure to 5 mM CHX for 90 minutes showing extensive structural damage to the cells. Magnification x 6,800. L - Light cells in both figures.

Figure 22. Low-magnification view of smooth muscle cells from tissues fixed after exposure to 5 mM CHX for 90 minutes, but the extent of damage is less in this tissue. Magnification x 6,800.



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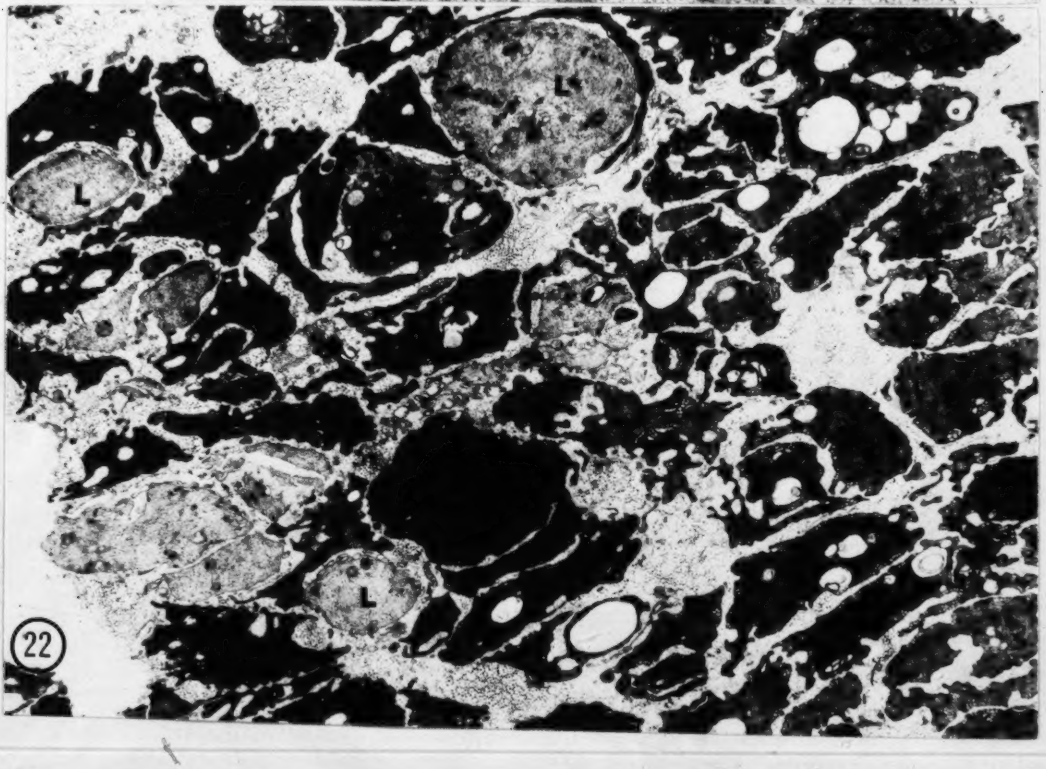

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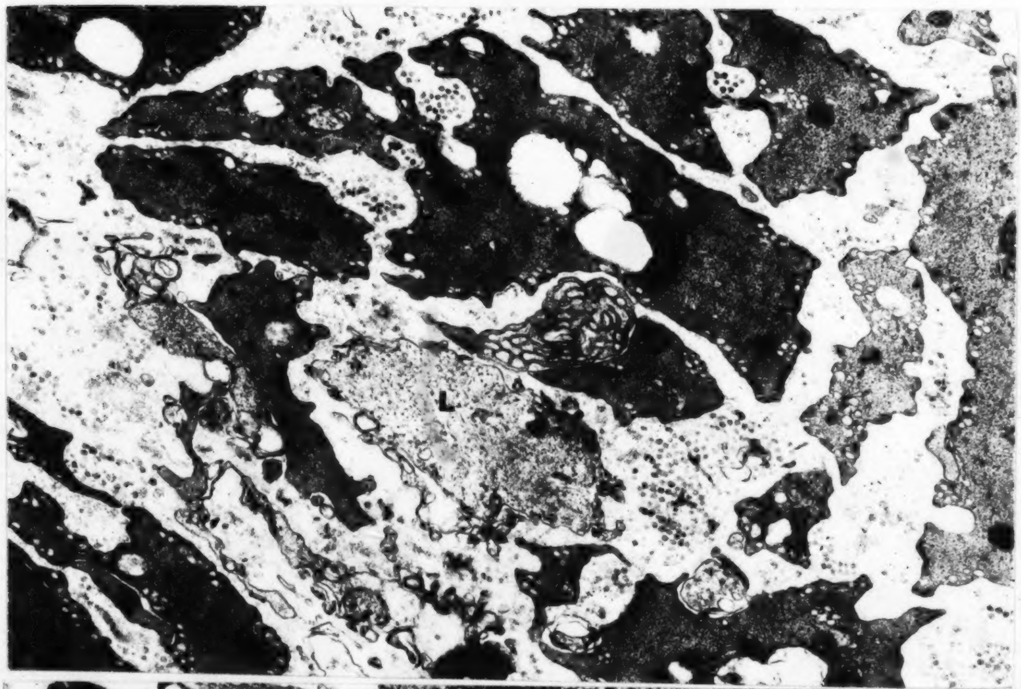
Figure 23. Smooth muscle cells from canine trachealis muscle fixed after pretreatment with CHX (5 mM) for 30 minutes and later exposure to 20 mM TEA + CHX for one hour. Magnification x 22,000.

L - Light cells.

Figure 24. Smooth muscle cells from canine trachealis muscle fixed after pretreatment with 5 mM CHX for 30 minutes and later exposure to 10 mM 4-AP + CHX for one hour. Magnification x 22,000.

L - Light cells.

G - Golgi cisternae.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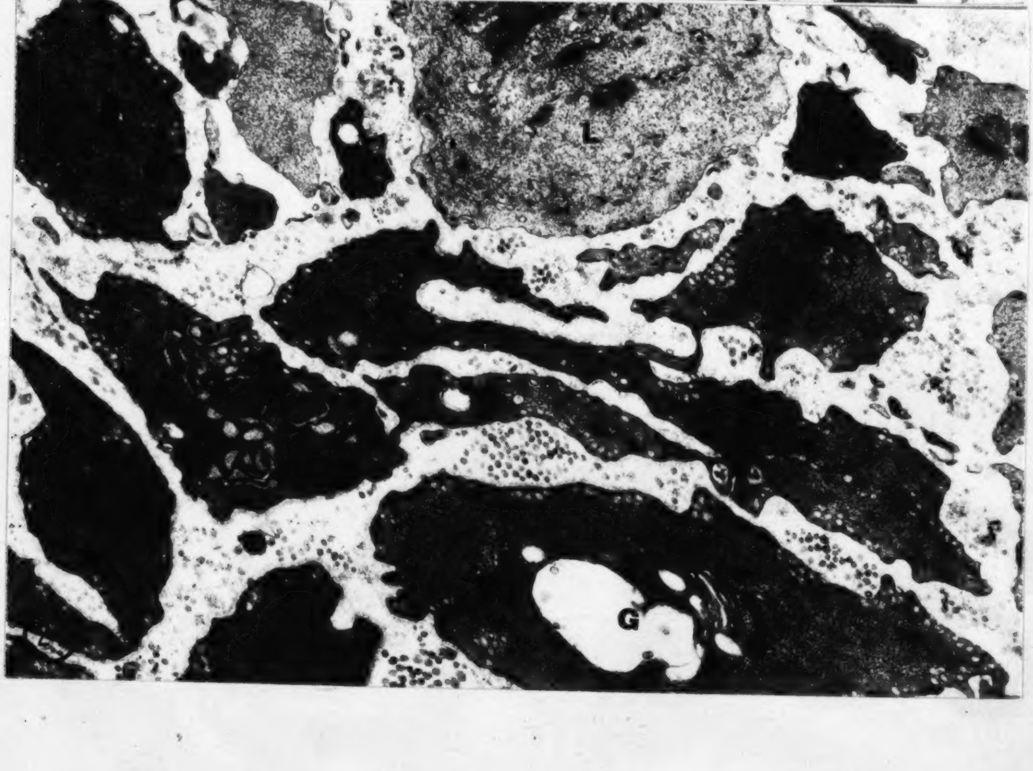
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TABLE VIII

Number of "Light" and Normal Cells in CHX-Treated Canine Smooth Muscle

n[a]	Total No. of Cells[†]	Normal Cells	"Light" Cells	"Light" Cells as % of Total
3[b]	738	418	320	43%
3[c]	1366	1177	189	13%

[†] Photographs of smooth muscle cells cut in cross-section were taken from one grid-square from each tissue at a magnification of 1900 and printed 3 times enlarged. The number of "light" cells and cells stained normally was counted from the photographs.

[a] Number of tissues studied.

[b] Where mechanical activity to 4-AP and TEA was abolished after preincubation with 5 mM CHX.

[c] Where mechanical activity to 4-AP and TEA was seen after preincubation with 5 mM CHX.

Design of experiments is in the text.

Effect of protein synthesis inhibition on formation of gap junctions:

ii) Number of gap junctions in control tissues: The mean number of gap junctions in control tissues ranged from 3.7 to 3.9 per 1000 μm length of membrane. There was no significant difference ($p > 0.05$) between these values and the number in tissues treated with 5 mM CHX alone for 90 minutes (the range was 2.9 to 3.8 per 1000 μm membrane length)(Table IX and X). In calculating these values, the membrane length of light cells were excluded from the measurements.

iii) Treated tissues: In the determination of the number of gap junctions after TEA and 4-AP treatment, the experiments were divided into two groups: a) those in which phasic mechanical activity was abolished after preincubation with CHX; and b) those in which such activity was seen.

Number of gap junctions in experiments with no mechanical activity following CHX:

a) The mean number of gap junctions in tissues treated with 20 mM TEA and 10 mM 4-AP for one hour respectively was: 6.7 and 7.2 per 1000 μm membrane length (Tables IX and X). These values were significantly higher ($p < 0.05$) than the number in the controls either with or without CHX treatment. Preincubation of tissues for 30 minutes with 5 mM CHX and later exposure to 20 mM TEA or 10 mM 4-AP for one hour prevented this increase in the number of gap junctions (4.3 for TEA and 3.8 for 4-AP per 1000 μm length of membrane). These

TABLE IX

Number of Gap Junctions in CHX- and 4-AP- and TEA-Treated Canine Tracheal Smooth Muscle[†]

Expt. No.	Control	Control + CHX	4-AP	4-AP + CHX	TEA	TEA + CHX
1	4.1	4.2	6.9	5.1	8.6	6.6
2	3.7	3.2	7.5	2.4	6.0	3.7
3	4.0	4.0	-	-	5.5	2.7
X	3.9	3.8	7.2*	3.8	6.7*	4.3

[†] Where mechanical responses to 4-AP and TEA were lost on preincubation with CHX.

Values are significantly higher ($p < 0.05$) than the values in all other tissues.

Consult the text for experimental design.

Results are analyzed as per methods described in Table III.

TABLE X

Number of Gap Junctions in CHX- and 4-AP- and TEA-Treated Canine Tracheal Smooth Muscle[†]

Expt. No.	Control	Control + CHX	4-AP	4-AP + CHX	TEA	TEA + CHX
1	3.8	2.8	19.3	11.2	7.2	5.1
2	3.0	2.2	8.3	6.6	6.8	3.7
3	4.3	3.6	-	-	10.4	9.5
X	3.7	2.9	13.8[*]	8.9[*]	8.1[*]	6.1[*]

[†] Where mechanical responses to 4-AP and TEA were present on preincubation with CHX.

[*] Values are significantly higher ($p < 0.05$) than the values in control and control + CHX-treated tissues.

Consult the text for experimental design.

Results are analyzed as per methods described in Table III.

values were significantly lower than those obtained for non-CHX-treated tissues exposed to potassium conductance blockers ($p < 0.05$) but not significantly lower ($p > 0.05$) compared to values in control tissues (Table IX).

Number of gap junctions in experiments with mechanical activity following CHX:

b) Tissues treated with 20 mM TEA or 10 mM 4-AP for one hour showed a significantly higher ($p < 0.05$) number of gap junctions (8.1 for TEA and 13.8 for 4-AP per 1000 μm length of membrane) than the number in control or control + CHX-treated tissues, as in Group a) above. Tissues preincubated with CHX for 30 minutes and later treated for one hour with either 20 mM TEA or 10 mM 4-AP showed a significantly higher number of gap junctions (6.1 for TEA and 8.9 for 4-AP per 1000 μm membrane length) than in control tissues exposed to CHX alone which was not observed with tissues showing no mechanical activity (Group a above). These values were slightly lower than the values in tissues exposed to the potassium conductance blockers, but not significantly so (Table X).

Discussion

The results of this study show that the rapid formation of gap junctions after treatment in vitro with 4-AP and TEA of canine tracheal smooth muscle is not prevented when more than 95% of the protein synthesis is blocked with CHX. This is true only in those tissues where the spontaneous phasic mechanical activity induced by the potassium conductance blockers is present after pretreatment with CHX. In tissues where mechanical activity was not induced, a significant increase in the number of gap junctions after TEA and 4-AP treatment was not observed (Table IX). Tissues pretreated with CHX and later exposed to TEA or 4-AP consistently showed fewer gap junctions than those treated with the potassium conductance blockers alone.

However, the results summarized above must be considered in the light of the effects of CHX treatment on structural damage to the smooth muscle cells. A greater proportion of damaged cells was encountered in tissues which failed to show any mechanical response to either TEA or 4-AP after CHX-preincubation (Table VIII). Gap junctions were not seen either between the damaged cells or between these and healthy smooth muscle cells in the immediate vicinity. The failure of mechanical response to TEA and 4-AP in some tissues after CHX-pretreatment could be due to the extent of injury to smooth muscle cells brought about by the inhibitor. The failure to get gap junction formation might also be related to the extent of cell damage produced by CHX.

The CHX-induced damage was not confined to the periphery of the tissues as far as could be determined from examination of thin-sections of treated tissues. The injured cells were stained lighter than the healthy cells. A detailed description of the damage resulting from CHX treatment is to be found in the Results section.

Light cells have been described in a variety of cells and under a variety of circumstances. In the epithelial cells of toad bladder, metabolic as well as mechanical injury has been shown to result in "light" cells (Crocker et al, 1970). Some of the structural alterations induced in these damaged cells, namely swollen endoplasmic reticulum, orthodox configuration of mitochondria , loss of membrane integrity, and decreased density of cells, resemble those seen in the tracheal smooth muscle after CHX treatment.

Somlyo et al (1971) have indicated that the density of smooth muscle cells is related to the extent of hydration before fixation for electron microscopy. A decreased electron density was observed in the smooth muscle of the rat uterus when injury increased cell water (Garfield and Daniel, 1976). On the other hand, hypertonicity caused an increased electron density of smooth muscle cells (Somlyo et al, 1971). The formation of light cells under these two conditions, however, is not accompanied by injury to smooth muscle cells to the extent that the caveolae are destroyed and the membrane continuity is lost with an attendant protein loss (Daniel and Robinson, 1971 a and b and Fay and Cooke, 1973 and Jones et al, 1973).

In a study on estrogen-stimulated rat uterus, Garfield and Daniel (1976) have shown that metabolic inhibition leading to ATP depletion as well as mechanical injury can result in formation of "light" cells. Myometrium from progesterone-treated and pregnant animals also had "light" cells. There was no evidence that the difference in density of smooth muscle cells was related to the state of contraction or relaxation of the tissue before fixation.

"Light" cells seen in CHX-treated canine tracheal smooth muscle could arise from injury leading to loss of membrane integrity, increased cell hydration, and loss of proteins. The presence of mitochondria in orthodox configuration, loss of surface caveolae and the swelling observed in light cells suggested the possibility of an altered metabolic state.

Gap junctions in CHX-treated tissues:

The continued formation of gap junctions in the absence of protein synthesis when cells were not severely damaged by CHX thus suggests that some proteins utilized to synthesize the extra gap junctions after TEA and 4-AP treatment are preformed. The various possibilities include:

i) De novo synthesis of membrane proteins is not necessary for rapid formation of gap junctions. Both TEA and 4-AP are capable of inducing junction formation within 10 to 15 minute exposure of the tissues to these drugs and this rapidity of action would tend to rule out the dependence of a phenomenon on new protein synthesis.

ii) CHX treatment did not result in complete inhibition of protein synthesis at the concentration employed and during the period of incubation. It is conceivable that, in the presence of the inhibitor, the residual protein synthesis which is resistant to inhibition could be involved in junction formation. This does not seem likely unless the proteins synthesized in the presence of the inhibitor are the junctional subunits themselves. We have no compelling evidence to support this possibility.

iii) There could be a differential sensitivity of tissues or cells to the effects of CHX. This was evident from the fact that the damage was confined to some of the cells. Whether the inhibition of protein synthesis brought about by CHX is a reflection of a generalized effect on all the cells or a variable degree of inhibition in different cells is difficult to resolve. My results, however, show that 95% of the cells were not injured although protein synthesis was inhibited to the same extent. This also speaks against the possibility that the less damaged cells really did not have protein synthesis inhibited.

The most likely possibility is that rapid junction formation is not dependent on new protein synthesis. The slight decrease in the number of gap junctions in the CHX-treated tissues could result if there is a limited quantity of pre-formed proteins involved in their formation or from an effect of CHX on the preformed gap junctions. Also, if many cells are damaged, the survivors may not have been able to make contacts with other surviving cells.

The formation of gap junctions in the absence of protein synthesis observed in canine trachealis has been previously shown in other systems as well. In a study by Epstein et al (1977) Novikoff hepatoma cells, dissociated in the presence of EDTA, have been shown to form gap junctions within a few minutes on reaggregation in culture. Neither inhibition of protein synthesis by CHX nor depletion of cellular ATP levels by iodoacetate (IAA) resulted in prevention of junction formation. There was no reduction in the percentage of coupled cells or in coupling coefficients between control and CHX-treated cells. CHX, at concentration used in these studies (100 µg/ml), produced 99% inhibition of protein synthesis over a 30 minute period.

On the other hand, a dependence of junction formation on de novo protein synthesis has been shown in some systems. In the chick embryonic heart cell suspensions, gap junction formation occurs during aggregation more than one hour after specific adhesion has taken place (Griepp and Bernfield, 1975). Formation of gap junctions seems to be related to acquisition of synchronous beating between the myocardial cells in culture. Cycloheximide inhibits this synchrony, suggesting that synthesis of new proteins is a prerequisite to formation of gap junctions in this system.

Gap junction formation during thyroxine-stimulated ependymoglial differentiation in tadpoles appears to depend on synthesis of proteins as well. Both CHX and Actinomycin D (which blocks transcription of the mRNA from DNA), if administered at a proper time after hormone treatment, block junctional development (Decker, 1976).

In both these cases, however, there was no evidence for a rapid formation of gap junctions in response to the specific stimuli. The delay in the appearance of junctions tends to lessen the probability of preformed membrane proteins being involved in their assembly. It wasn't surprising therefore that the formation of gap junctions was dependent on new protein synthesis. The hormone-dependent differentiation in tadpoles seems to be dependent, in addition, on synthesis of the messenger.

The lack of sensitivity of rapid gap junction formation to inhibition of protein synthesis in canine trachealis as well as in Novikoff hepatoma cells seems to suggest a mechanism involving reaggregation or assembly of subunits. The nature of the subunits in smooth muscle is not known, but studies on the gap junctions isolated from mouse and rat liver reveal that they are made up of two major protein components (Goodenough, 1974). The term connexin has been given to these proteins. Assembly of these subunits into structures recognized as gap junctions in thin sections is a likely mechanism by which cells can regulate their number in response to physiological or pharmacological interventions. A detailed discussion of the aspects of such subunit interactions is dealt with in Chapter V (Discussion section).

CHAPTER V

FREEZE-FRACTURE STUDIES OF CANINE TRACHEAL SMOOTH MUSCLE

CHAPTER V

Freeze-Fracture Studies:

Introduction

The studies reported in the previous chapters indicate that gap junctions can be demonstrated in the canine tracheal smooth muscle fixed *in vitro* by techniques which give adequate preservation of cells. The number of gap junctions is increased on treatment of the tissue with TEA or 4-AP and this increase is not prevented when new protein synthesis is blocked by CHX.

The gap junctions in appropriately oriented thin sections of tissues have a typical 7-layered structure with a central gap about 2 nm wide (Figures 3 and 4). The junctions were typically found connecting processes of cells. This arrangement was seen even in control tissues which never contracted spontaneously *in vitro*. Both TEA and 4-AP are capable of inducing gap junction formation *in vitro* very rapidly (< 10 minutes).

Freeze-fracture studies reveal that the gap junction is a highly specialized cell-to-cell contact (see McNutt and Weinstein, 1973). Such studies have been carried out in a variety of tissues to understand the fine structure of the gap junction as well as to study the stages in the formation of such contacts between cells (see Discussion).

The objectives of the present investigation are:

i) To attempt to demonstrate gap junctions in the canine tracheal smooth muscle by the freeze-fracture technique;

ii) To understand the possible mechanism underlying the rapid formation of gap junctions in tissues treated with TEA and 4-AP *in vitro* using this technique;

iii) To evaluate the possible usefulness of this technique in quantitative studies of gap junctions in this tissue.

Materials and Methods

Tissue preparation:

Canine tracheal smooth muscle was obtained using the Methods described in Chapter II. Control smooth muscle strips were incubated *in vitro* in Krebs solution at 37° C for one hour and fixed in 2% glutaraldehyde in 0.075 M cacodylate buffer (pH 7.4) containing 4.5% sucrose. Some tissue strips were incubated in either 33 mM TEA- or 10 mM 4-AP-containing solution for one hour at 37° C and fixed the same way as control tissues.

After an initial two hour fixation, the tissues were rinsed with 0.1 M cacodylate buffer (pH 7.4) for at least one hour. The tissues were then incubated at room temperature (22° C) in a solution of 25% glycerol (V/V) in 0.05 M cacodylate buffer of the same pH for at least 90 minutes.

Procedure for freeze-fracturing:

The tissue strips were dissected under a dissecting microscope into very small segments. Care was taken to ensure that the tissues were kept moist during this procedure. Small gold specimen-holders were filled with the tissue pieces and rapidly frozen in liquid Freon-22 cooled by liquid nitrogen and finally immersed in liquid nitrogen.

Specimen transfer:

A Balzers BA 360M Freeze-etch instrument was used. The specimen stage was cooled under vacuum with liquid nitrogen to a temperature of -170° C. The chamber was vented to atmospheric pressure before specimen transfer. The specimen stage was painted with liquid Freon-22 and the specimen-holders containing the tissue were quickly transferred and the chamber evacuated.

Fracturing:

The tissues were fractured at -100° C with a cold knife at ~ 1.5×10^{-7} torr vacuum. When the specimens were fractured uniformly, replication was carried out.

Replication:

The fractured surfaces were coated with Pt-Carbon for 8 seconds at an angle of 45° and then coated with Carbon for 10 seconds at an angle of 90°. After covering the specimens with the knife, the chamber was vented. The specimen-holders with the replicated tissues were immersed in 40% chromic acid.

Replica retrieval:

After immersion in 40% chromic acid overnight to digest the tissues, the floating replicas were aspirated into distilled water. After washing once, the replicas were transferred to a solution of commercial bleach (Javex) for about two hours. They were later washed with distilled water twice and picked up onto 300-mesh copper grids coated with collodion-carbon. The replicas were examined in JEM-7A or Philips EM-301 electron microscopes.

Freeze-fracture nomenclature:

The fracture faces of tissues in the replicas will be described in the text using the nomenclature suggested recently (Branton et al, 1975).

Results

The technique consists of two distinct processes: freeze-fracturing and freeze-etching. Fracturing a tissue results in the breakage along planes of weak bonding in the membrane or elsewhere and etching sublimes volatile materials, usually water, from the non-volatile materials of the specimen (see Discussion). Fracturing is an essential part of the technique, while etching is an optional procedure. In the present study, etching was not attempted. Any etching which might have taken place was done inadvertently.

Gap junctions in the freeze-fracture replicas:

The results using thin-section electron microscopy show that canine trachealis incubated in Krebs solution *in vitro*, has gap junctions. Their mean number was 3.36 per 1000 μm membrane length. In TEA-treated tissues, the number of gap junctions ranged from 5.04 to 7.40 per 1000 μm membrane length and in 4-AP-treated tissues from 7.71 to 10.80 per 1000 μm membrane length. It was also evident that the smooth muscle cells had very irregular shapes with several processes and the gap junctions were almost exclusively found on processes connecting two cells. The abutment-type of gap junction was not seen in canine trachealis.

Examination of freeze-fracture replicas of canine trachealis revealed that gap junctions were very rarely encountered. The ones seen were also on cell processes. Some of the gap junctions were seen in the interdigitations of smooth muscle cells.

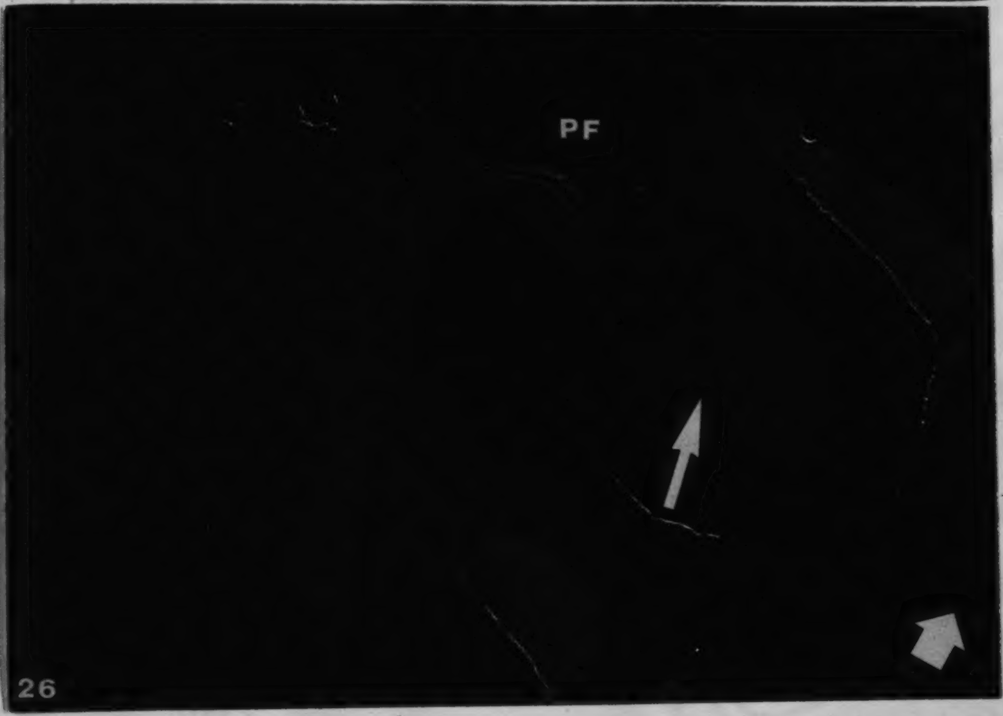
Figure 25 shows a gap junction on a cell process in the freeze-fracture replica obtained from a control tissue. The structure appears as a cluster of particles on the membrane PF fracture face.

Figure 26 shows a gap junction on the membrane PF fracture face in a replica obtained from a TEA-treated tissue. The structure is also located on a cell process and shows a hexagonal array of

Figure 25. Freeze-fracture replica of canine trachealis (control tissue). A gap junction (arrow) seen as a cluster of particles on the membrane PF face of a cell process. Magnification x 150,000.

Shadow angles are indicated by arrows (bottom right-hand corner) in all the freeze-fracture micrographs.

Figure 26. Replica from tissue treated with 33 mM TEA for one hour. The gap junction (arrow) shows an array of particles on the membrane PF face of a cell process. Magnification x 100,000.



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particles.

Figure 27 shows the freeze-fracture replica from a 4-AP-treated tissue. The fracture is through a series of interdigitations of two adjacent smooth muscle cells, exposing both the PF and the EF membrane faces. At some places, the membrane has been cross-fractured. A gap junction can be seen as aggregation of membrane particles on the PF face.

A total of 15 replicas each from control, TEA-treated, and 4-AP-treated tissues from 13 animals were examined in this study. The average diameter of the junctions in the freeze-fracture replicas was 0.15 μm when measured at the largest diameter.

Freeze-fracture appearance of membranes:

The freeze-fracture replicas obtained from tissues contained extensive membrane fracture faces. There were numerous particles distributed in the membranes. The PF face usually contained a greater density of particles than the EF face. The surface caveolae were arranged in linear rows in the membrane. However, no gap junctions were seen in the membrane fracture faces, except on cell processes.

Figure 28 shows a membrane PF fracture face with the caveolae and many membrane-intercalated particles.

Figure 29 presents a membrane EF fracture face with the caveolae arranged in linear arrays. The density of particles is less compared to that in the PF face. The density of particles was higher in regions surrounding the caveolae than in other regions of the membrane

Figure 27. Freeze-fracture replica from tissue treated with 10 mM 4-AP for one hour. A gap junction (arrow) is seen on the membrane PF face. Magnification x 100,000.

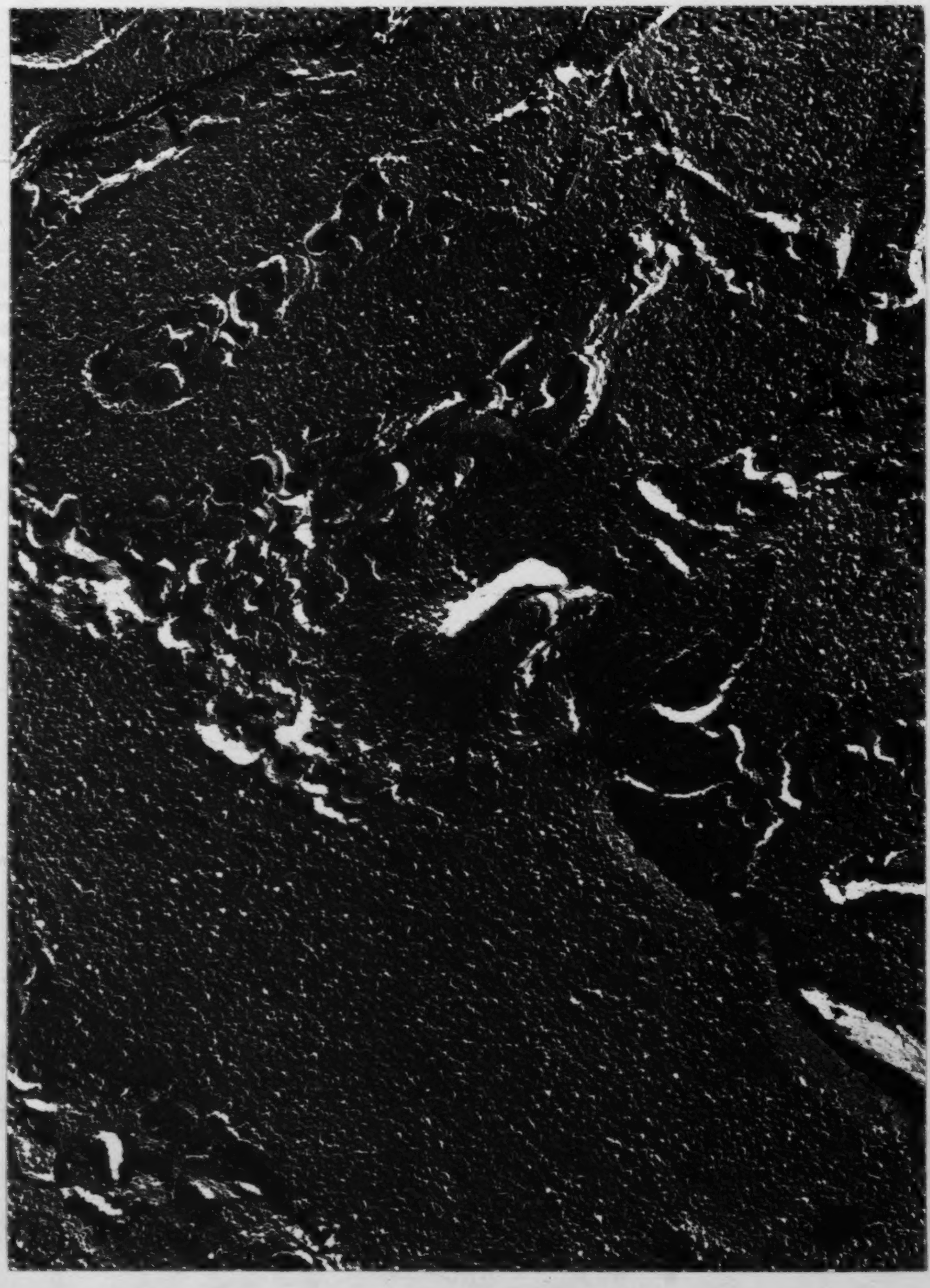
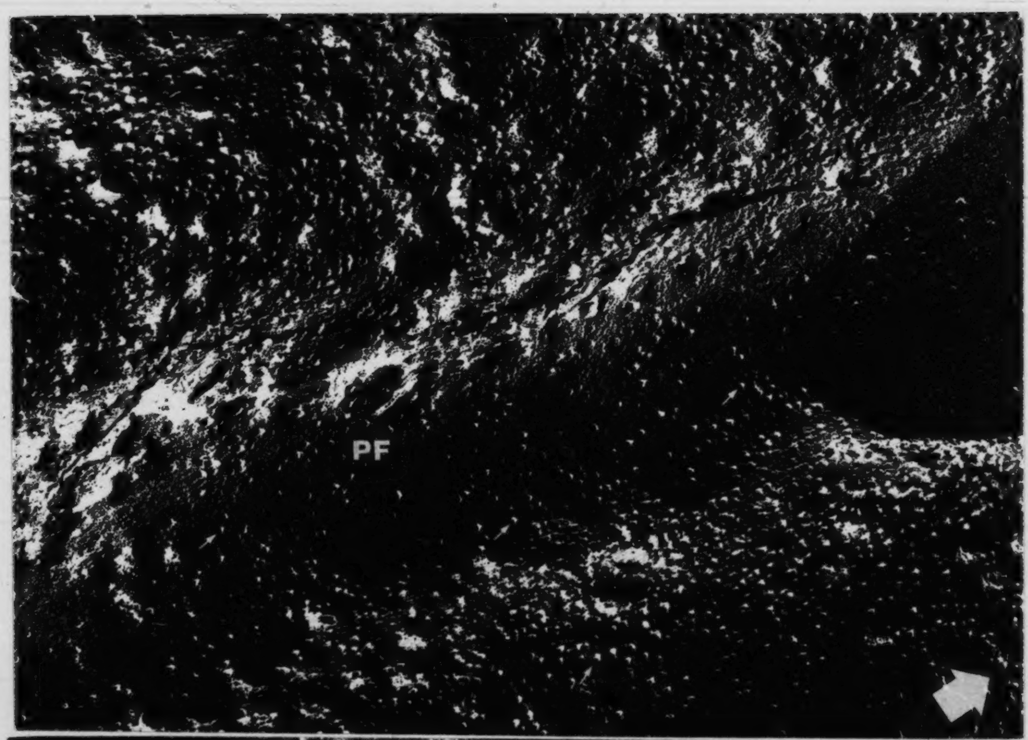
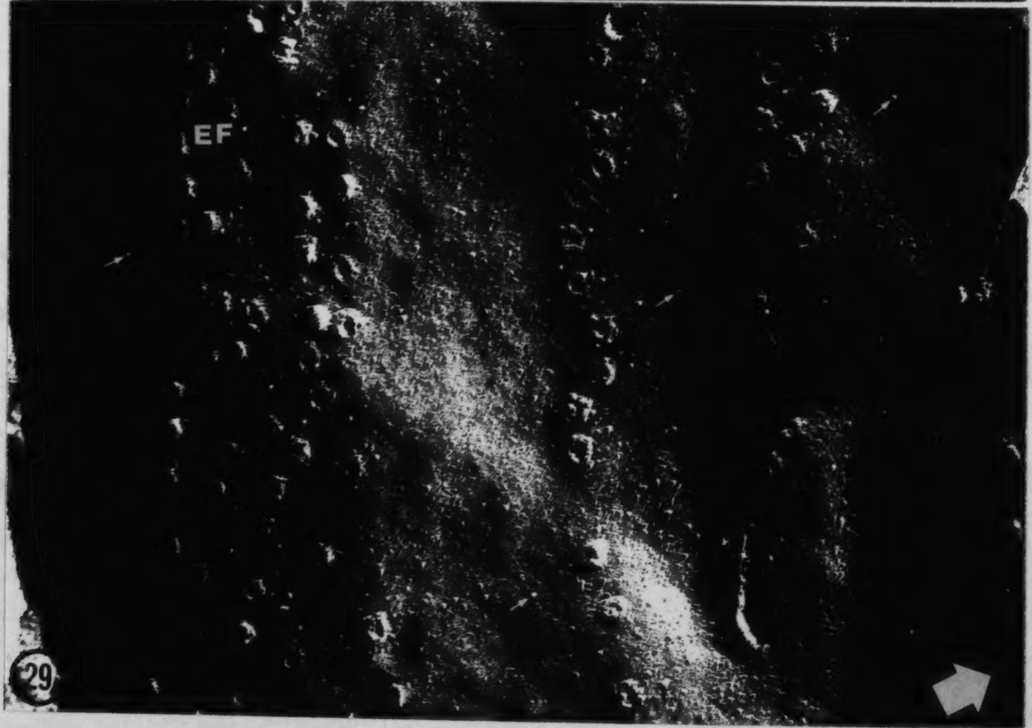


Figure 28. Shows a membrane PF face with numerous particles (small arrows). The caveolae are arranged in rows. Magnification x 114,000.

Figure 29. Membrane EF face with caveolae arranged in rows and particles. Magnification x 71,000.





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on the PF face. There are no gap junctions in both fracture faces of membranes in these replicas as well as in others examined. There were also no small gap junctions (see Discussion) in the membrane fracture faces. These small gap junctions, if present, would not be recognizable in thin sections of tissues.

The fracture plane does not always proceed through the hydrophobic region of the membrane. Sometimes there was a cross-fracture of the membrane where it appeared as a ridge. The caveolae in such replicas are also cross-fractured (Figure 27).

Figure 30 shows a replica of tissue in which some of the cytoplasmic organelles are fractured in addition to the plasma membrane.

Discussion

Freeze-fracture technique:

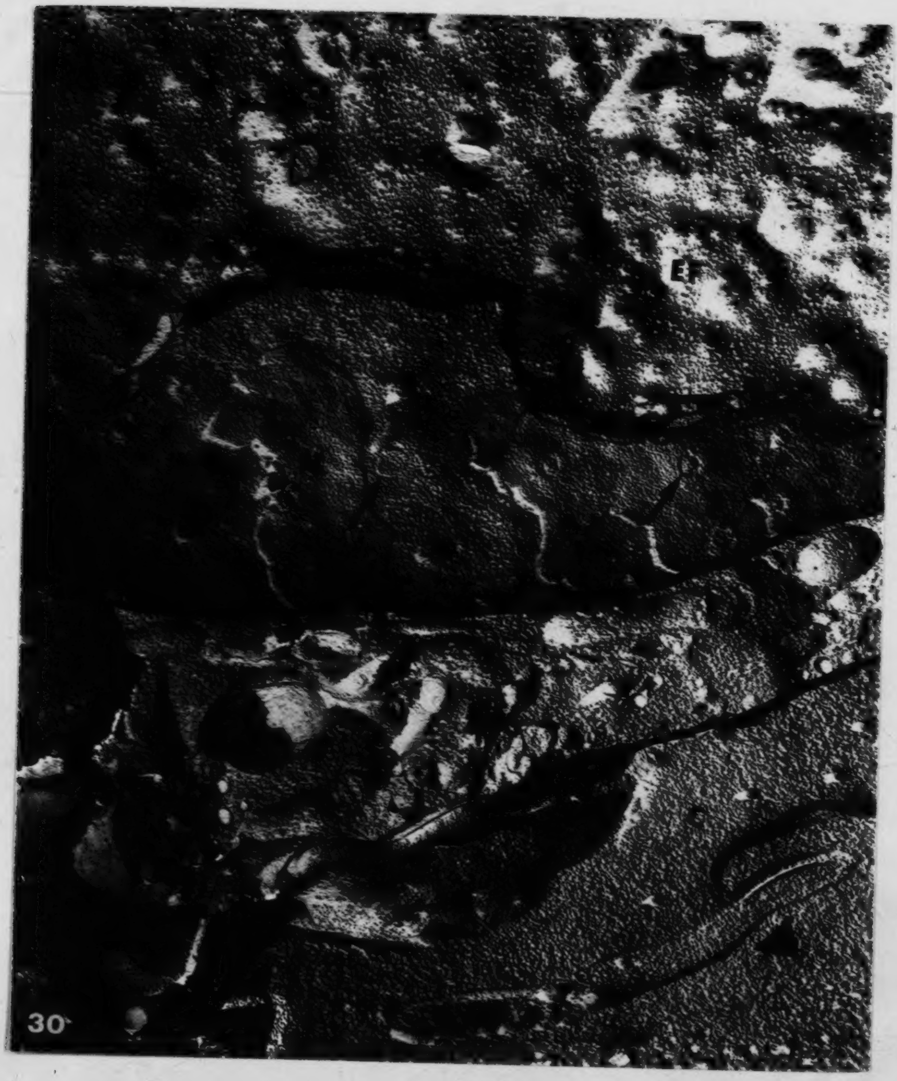
The technique of freeze-fracturing was first applied to biological tissues by Steere (1957) and later developed by Moor *et al* (1961). The technique consists of two separate processes: fracturing and etching. Freeze-fracturing usually results in the breakage along planes of weak bonding in the membrane; and etching sublimes away volatile materials, usually water, from non-volatile materials of the tissue. Fracturing is an essential aspect of the technique, while etching is done as an option (Davy and Branton, 1970).

Figure 30. Freeze-fracture replica of canine trachealis. Magnification x 32,000.

EF - membrane EF fracture face.

N - fracture faces of nuclear membranes with the nuclear pores (arrows).

C - cytoplasm of cell.


EF
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Interpretation of freeze-fracture replicas:

Earlier interpretations of freeze-fracture replicas of biological specimens suggested that the fracture plane passed along the true surfaces of membranes (Branton and Moor, 1964 and Moor, 1964). Although this interpretation was accepted initially, it soon became apparent that many freeze-fracture images could not be so explained. Branton (1966 and 1967) suggested that the cleavage plane passed along the membrane interior resulting in formation of two lamellae. A body of evidence accrued in later studies (Deamer and Branton, 1967 and Branton, 1967 and Chalcroft and Bullivant, 1970 and Wehrli et al, 1970 and Weinstein et al, 1970 and Pinto da Silva and Branton, 1970 and Tillack and Marchesi, 1970 and Sleyter, 1970) supports Branton's interpretation. The most direct evidence to this suggestion came from the double-replica method (Steere and Moseley, 1969 and Wehrli et al, 1970 and Weinstein and McNutt, 1970 and Chalcroft and Bullivant, 1970) in which both the lamellae could be replicated, retrieved and examined for complementarity.

The two lamellae resulting from the cleavage and their surfaces were identified and named (McNutt and Weinstein, 1970). The lamella which remains attached to the cytoplasm of the cell was called LM1 and the one attached to the extracellular space as LM2 by these authors. Thus, the fractured surfaces exposed are not the true surfaces but new surfaces produced from within the interior of the membranes. These new surfaces are called "faces" in order to distinguish them from the true membrane surfaces.

The cleaved face of the lamella which remains attached to the cytoplasm is called Face A and the one adjacent to the extracellular compartment Face B. Face A can be visualized while looking from outside the cell inward and Face B from within the cell outward. The terms convex and concave fracture faces refer respectively to the membrane A and B faces. When etching is done in conjunction with fracturing, the true surfaces can be visualized in the resulting replicas.

The fracture faces referred to in this study conform to a nomenclature proposed recently (Branton et al, 1975). Membrane fracture face A is referred to as PF face and the fracture face B as EF face. The corresponding etched surfaces are referred to as PS and ES surfaces respectively.

Freeze-fracture appearance of membranes:

The biological membranes in freeze-fracture replicas appear as extended smooth sheets interrupted by many "particles". The PF and EF fracture faces in many membranes appear to have a different density of these particles, suggesting asymmetry of membrane structure (Branton, 1969). Examination of freeze-fracture replicas of biological membranes also revealed that the number of particles was highest in the physiologically active membranes like the chlorplast lamellae (Branton and Park, 1967), erythrocyte membrane (Weinstein and Bullivant, 1967), etc. On the other hand, their number was least in inactive membranes such as myelin (Branton, 1969). This comparison suggested the possibility that such particles could be proteins. Engstrom (quoted by Branton, 1971) showed that the particles seen in red blood cell membranes disappeared on treatment with pronase, a proteolytic enzyme. Correlated freeze-etch and X-ray diffraction studies on certain model phospholipid membranes suggested that the smooth regions of membranes represented fracture through the lipid regions (Deamer et al, 1970). Since there was no evidence that the particulate nature of biological membranes could arise from bulk phase lipids, it was suggested that the particles might be the intrinsic proteins or specific protein-lipid interactions in membranes.

Experiments with lamellar phase lipids or lipid extracts from the erythrocyte ghosts and sarcoplasmic reticulum (SR) suggested that the fracture faces were devoid of particles (Deamer et al, 1970). On the other hand, fracture faces of lipid vesicles and lamellar lipid phases which had been reconstituted with membrane proteins from the ghosts or SR contained particles (MacLennan et al, 1971). Freeze-etching studies also revealed that the particles on the erythrocyte membranes contained receptors for influenza virus and wheat germ agglutinin (Tillack et al, 1971), as well as the A and B antigens (Pinto da Silva et al, 1970). Thus, the membrane particles in these studies must include glycoprotein or glycolipids.

The particles have been shown to be capable of translational movement along the plane of the membrane. In the erythrocyte ghosts, aggregation of particles can be induced by lowering the pH of the medium and this is reversed by prefixation in glutaraldehyde or incubation in media of high ionic strength (Pinto da Silva, 1972).

A role for microtubules and microfilaments in the lateral mobility of membrane-intercalated particles has been proposed (Nicolson et al, 1971). The cell membrane is considered as a solution of integral membrane proteins in a fluid lipid bilayer (Singer and Nicolson, 1972). In response to specific stimuli, either arising extracellularly or intracellularly, rapid lateral mobility of some proteins could occur. Cells do possess mechanisms that control the mobility of membrane components through associations to cytoskeletal elements like microtubules, microfilaments and intermediate filaments. This is evident in ligand-receptor movements resulting in phenomena such as receptor "capping" in lymphocytes, cell attachment and movement, endocytosis, etc.(see Nicolson et al, 1976).

The evidence for translational mobility of membrane proteins in the lipid bilayer is overwhelming. The evidence that membrane particles seen in freeze-fracture replicas are indeed the membrane proteins is however indirect. The possible physiological significance of particle movements resulting in aggregation is discussed below.

Fine structure of gap junctions:

The fine structure of gap junctions as revealed by thin-section, negative-staining and freeze-fracture techniques has been discussed (see Chapter I). In freeze-fracture replicas, the gap junctions reveal closely packed particles arranged in a hexagonal array with a centre-to-centre spacing of 90 to 100 A° when viewed on the membrane PF face and corresponding array of depressions on the membrane EF face. The particles in the non-junctional membrane, on the other hand, tend to be randomly distributed.

Freeze-fracture studies on the canine tracheal smooth muscle:

The freeze-fracture studies on canine tracheal smooth muscle demonstrate the presence of gap junctions between cells, thus confirming my thin-section EM studies. The gap junctions seen on the replicas appear in different forms. Some of the gap junctions appeared as a cluster of particles on the membrane face while others appeared as aggregations of particles in a hexagonal array. All the gap junctions thus far encountered were on fracture faces of cell processes.

The membrane fracture faces had numerous particles distributed with no discernible order. The PF fracture face had a greater density of these particles than the EF face. The particle density was also higher in regions surrounding the surface caveolae than in other regions of the membrane. There were no gap junctions on the membrane faces. In contrast to my thin-section results, there were very few gap junctions in replicas of both control and TEA-and 4-AP-treated tissues.

There was no evidence for the occurrence of small gap junctions in the membrane fracture faces examined. We expected that any small gap junctions, if present, would be more likely to be seen in freeze-fracture replicas than in thin sections. The minimum number of subunits required to act as a low-resistance pathway for current flow in smooth muscle is not known. It is also not known if subunit arrangements into patterns, other than the more familiar hexagonal packing, could be called as gap junctions. Also it is not clear that an aggregation of particles in membrane of one cell corresponds to a similar aggregation in an apposed cell.

In a recent freeze-fracture study of nexuses in a variety of smooth muscle tissues (Fry et al, 1977), it has been observed that "nexuses" occur in various shapes and sizes. The authors speculate that aggregation of two or three subunits in the membrane could provide a region of low-resistance between the cells. A preponderance of small gap junctions were seen in the smooth muscle of the guinea-pig sphincter pupillae. Gap junctions as small as 0.001 μm^2 were reported, although there were no micrographs showing such structures. The particles constituting the subunits in a nexus have been characterized in isolated gap junctions from liver (Duguid and Revel, 1975). At present the chemical composition of the particles seen in the non-junctional membrane is not known and there are no morphological features by which they can be distinguished from the particles of a nexus. Thus, it is quite arbitrary to designate any aggregation of particles as nexuses.

Formation of gap junctions as studied by the freeze-fracture technique:

Gap junction formation between cells in vivo and in culture has been studied in a variety of systems (Revel et al, 1973 and Johnson et al, 1974 and Decker and Friend, 1974 and Benedetti et al, 1974 and Albertini and Anderson, 1974 and Decker, 1976) using the freeze-fracture technique. The stages in the formation of these junctions, as revealed in the replicas have been already discussed (Chapter 1). The manner in which the gap junction particles, once inserted into the membrane, are aggregated into a polygonal lattice of subunits is not known. A role for microtubules or microfilaments in such an assembly has been suggested (Johnson et al, 1974).

The TEA- and 4-AP-induced rapid formation of gap junctions in the canine trachealis has been shown to be independent of new protein synthesis (see Chapter IV). This suggests that the subunits involved in their assembly are preformed. The subunits would be arranged into larger gap junctions which are demonstrable by thin section techniques. We could not detect any formation plaques in the replicas examined. Since gap junctions were located exclusively on processes of cells and because of the difficulty in getting fracture faces of such processes, the various stages underlying the rapid formation of gap junctions in this tissue could have been missed.

CHAPTER VI

REVIEW AND CONCLUSIONS

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A. Ultrastructure of Canine Tracheal Smooth Muscle

i) Gap junctions: The results of this investigation have established the presence of gap junctions between smooth muscle cells of the canine trachea. Gap junctions were demonstrated, in appropriately oriented sections, as 7-layered structures, formed by the apposition of membranes of two neighbouring cells, with a central gap about 2 nm wide. These junctions were found exclusively between cell processes. Junctions were also seen occasionally between processes of the same cell. Gap junctions could also be demonstrated in tissues fixed *in situ*. This rules out the possibility that they could form when tissues are incubated *in vitro*. The technique of tissue fixation was found to be adequate in demonstrating gap junctions and in good preservation of other cellular structures.

Tissues were fixed initially with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and post-fixed in 1% osmium tetroxide in 0.05 M cacodylate buffer of the same pH. They were usually stained *en bloc* with saturated uranyl acetate and processed for thin section electron microscopy.

The claim that glutaraldehyde fixative is unsuitable for preserving gap junctions (Dewey and Barr, 1962) is contradicted by my study. Thus, in canine trachealis, which has been described as a multiunit smooth muscle, gap junctions are consistently seen. The claim that such smooth muscles lack gap junctions has also been contradicted from the results of this investigation.

ii) Innervation of the canine trachealis: This study reveals the presence of a cholinergic excitatory as well as an adrenergic inhibitory innervation to the canine tracheal smooth muscle. Two types of varicosities were characterized on the basis of the distribution of vesicles: those containing predominantly small agranular and a few large granular ones; and varicosities containing a preponderance of small granular vesicles. The varicosities containing small and large granular vesicles were not seen associated with blood vessels in the tissues examined. Suzuki *et al* (1976) have described the innervation of canine trachealis similar to what I found in these studies. In addition, their histochemical staining techniques revealed the presence of acetylcholinesterase and catecholamine-fluorescence.

iii) Electrical stimulation: The results of the effects of electrical field stimulation on the mechanical activity of canine trachealis revealed the presence of functional excitatory innervation, whose effects were blocked by atropine. This suggested that the underlying mechanism was through release of acetylcholine from the intrinsic nerve-endings. Furthermore, when the tone was raised by TEA treatment, electrical stimulation of the tissues resulted in relaxation, which was blocked by β-adrenergic antagonist propranolol. After propranolol, the response consisted of a contraction. The existence of an adrenergic inhibitory mechanism to this smooth muscle was established from these studies.

Suzuki et al (1976) found no evidence for a non-adrenergic and non-cholinergic inhibitory system in the canine tracheal smooth muscle. Evidence for the presence of such an inhibitory innervation has, however, been obtained in the guinea-pig and human tracheo-bronchial smooth muscles (see Review of literature). Thus, there seem to be species differences regarding the types of innervation.

iv) Effects of TEA: Canine tracheal smooth muscle does not exhibit phasic mechanical activity in vitro. However, changes in tone in vivo have been reported (Loofbourrow et al, 1957). Kroeger and Stephens (1975) and Suzuki et al (1976) have reported stable membrane potential with no evidence for spontaneous rhythmic depolarizations in the smooth muscle cells of canine trachea. Treatment with TEA was shown, in their studies, to result in depolarization with phasic electrical and mechanical activities. The rectifying property of the membrane was abolished by TEA. Kroeger and Stephens (1975) reported an increase in the space constant from 1.6 mm to 2.8 mm after treatment with TEA. However, Suzuki et al (1976) reported a longer space constant for the control tissue (3.2 mm) as well as a time constant value of 450 msec. Thus, this smooth muscle has cable properties as studied in vitro. The reason for the lack of initiation of spontaneous spike activity was ascribed to a high resting permeability to K^+ in this tissue. TEA, by blocking the resting K^+ conductance and abolishing the rectification, was thought to result in depolarization and a weak activation of Na^+ conductance.

The TEA-induced mechanical activity consisted of two components: an increase in tone as well as initiation of phasic activity. The maximum active tension was dose-related. The time to reach steady-state tension was dose-related as well. The phasic activity was not sensitive to atropine, suggesting that the effects were not mediated through release of acetylcholine from the nerve-endings. Atropine diminished the tone, but the tone recovered with time. The mechanical response to TEA could be blocked by D-600, an agent which blocks Ca^{++} entry in smooth muscle cells upon depolarization. In Ca^{++}-free Krebs solution containing 0.5 mM EGTA, the tissues failed to show any mechanical response to TEA treatment. Upon addition of Ca^{++} to the medium, the mechanical activity was restored. Thus, the TEA-induced mechanical activity seems to depend on availability of extracellular Ca^{++}. Kroeger and Stephens (1975) showed that the myogenic response in the canine trachealis was dependent on extracellular Ca^{++} and was abolished by D-600. It is not certain whether the initiation of mechanical activity or myogenic response by TEA in this smooth muscle is the result of an inward Ca^{++} current resulting in depolarization. The Ca^{++} influx associated with the mechanical response could result from depolarization of the cells by TEA.

Effects of TEA on gap junctions:

The TEA-induced changes in the biophysical properties of this smooth muscle are accompanied by changes in the number of gap junctions. The rapid increase in the number of gap junctions is seen at a time when spontaneous activity is also initiated, suggesting a correlation in time. However, there was no difference in the ability of various doses of TEA to increase the number of gap junctions even though the mechanical response was dose-related.

The presence of gap junctions in this smooth muscle could provide one basis of coupling between cells and their increase after TEA treatment could provide better coupling. Although the measured diameters of the gap junctions in the treated tissues were not different from those in control tissues, the increase in their number should increase the gap junctional membrane area.

v) Effects of 4-AP on the canine trachealis: 4-AP was also shown to result in initiation of phasic mechanical activity of canine tracheal smooth muscle. The effect of 4-AP on tension was almost immediate. The activity consisted initially in a rise in tone followed by oscillations of tension. The maximum active tension was found to be dose-related. Though there was fluctuation of tension in 4-AP-treated tissues (as with TEA-treated tissues), it did not return to the original base-line.

The effects of electrical field stimulation of tissues after treatment with different doses of 4-AP revealed that the relaxation due to field stimulation was evident only after exposure to higher doses of the drug. This relaxation was shown to be sensitive to propranolol. After propranolol, field stimulation caused a contractile response.

The compound 4-AP was shown to selectively block potassium channels in a variety of excitable membranes, although some important differences were elucidated in its mechanism of action from that of TEA (see Review of literature).

The mechanical effects of 4-AP were completely blocked by atropine. This suggested that the effects were due to release of acetylcholine from the intrinsic nerve-endings. After atropine treatment, phasic activity could not be initiated after increasing the tone by electrical stimulation.

Exposure of tissues to acetylcholine caused a sustained increase in tone which reached a plateau and remained unaltered. There was no phasic mechanical activity after acetylcholine treatment. Atropine blocked this tone. If the effects of 4-AP on phasic activity were entirely dependent on release of acetylcholine from nerves, then application of acetylcholine should have resulted in phasic activity. Since this was clearly not the case, it could be argued that phasic activity was not dependent on release of transmitter. The other possibilities are either dependence of phasic activity on previous tone in the tissue or a phasic release of transmitter from the nerves.

Effect on gap junctions:

Tissues treated with 4-AP showed an increase in the number of gap junctions compared to untreated control tissues. The ability of this compound to induce junction formation was found to be rapid since the structural effects were seen as early as 10 minutes after exposure of the tissues. The different doses of the drug resulted in similar increases in the number of gap junctions and at the same time there was a correlation between the time of onset of phasic activity and formation of gap junctions. But a correlation between dose of 4-AP and increase in gap junctions, as in tension response, did not occur.

Treatment with acetylcholine of tissues did not result in an increase in the number of gap junctions. Tissues pre-treated with atropine to block the mechanical effects and later exposed to 4-AP still showed an increase (although smaller) in their number. These results rule out the dependence of rapid formation of gap junctions on the release of acetylcholine as well as on tone. Thus, the effects of 4-AP should be the result of a direct smooth muscle action to cause an increase in the number of gap junctions.

After exposure to 3 mM 4-AP for one hour (when phasic activity was established), tissues from three animals were washed with Krebs solution until the tension reached the base-line. These tissues were kept in Krebs solution for an additional one hour period and fixed and examined for the number of gap junctions. The number of gap junctions in these tissues was still higher than in untreated control tissues (10.8 ± 1.25).

Formation of gap junctions in 4-AP-treated tissues in the absence of mechanical activity, either after atropine pre-treatment or after washing with Krebs solution for one hour, indicates that either single-unit behaviour is not necessary for this process or gap junction formation is not sufficient for initiation of single-unit behaviour in the tissue. However, there is no evidence for single-unit behaviour of this tissue in the presence of acetylcholine.

vi) Role of gap junctions in cell-to-cell coupling: Canine tracheal smooth muscle has cable properties as studied in vitro. The length constant of the tissue is many times the average cell length. The evidence for cable-like behaviour of this tissue is that there is exponential decay of applied current over distance from the stimulation site. TEA causes an increase in the length constant of this tissue. This effect was interpreted by Kroeger and Stephens (1975) as the result of an increase in the transmembrane resistance accompanying the TEA effect to decrease potassium conductance. They assumed that the coupling and the internal core resistance remained unchanged.

Gap junctions are thought to be the structural bases for current-flow between smooth muscle cells, although there is no direct evidence to this hypothesis. Many coupled smooth muscles have been shown to have gap junctions. On the contrary, there are coupled smooth muscles which do not have gap junctions. Daniel et al (1976) proposed that gap junctions could be sufficient for electrical coupling, if present, but not essential. Cell-to-cell contacts of various types exist between smooth muscle cells and some of these should be considered as potential candidates as low-resistance contacts. Since gap junctions are present in canine tracheal smooth muscle, they could provide one basis for electrical coupling between the cells. Their rapid formation after TEA- and 4-AP-treatment presumably provides better coupling. The resultant increase in the gap junctional area should result in current spread over a greater distance between the cells. This hypothesis, though attractive, remains speculative until gap junctions are established to be the low-resistance contacts in smooth muscle.

vii) Mechanisms underlying the rapid formation of gap junctions: Potassium conductance blockers like TEA and 4-AP were shown to result in rapid formation of gap junctions in the canine tracheal smooth muscle. This rapidity of action prompted me to investigate further the mechanisms underlying their formation.

The chemical composition of gap junctions in smooth muscle is not known. Studies on isolated gap junctions from livers of mice and rats (Goodenough, 1974) reveal that they are made up of specific proteins, described as connexins. If these proteins form the subunits of gap junctions, the question arises as to what mechanism controls their formation, transport to the plasma membrane and organization in the membrane into a specific pattern recognized as gap junctions in thin section EM or by freeze-fracture techniques.

I considered two possibilities underlying the rapid synthesis of gap junctions induced by TEA and 4-AP in the canine trachealis:

a) Increased synthesis of the membrane proteins involved in their assembly; and/or

b) Increased aggregation of pre-formed subunits into structures recognizable in thin sections as gap junctions.

Dependence of junction formation on new protein synthesis:

a) Incorporation of ^{3}H-leucine into TCA-insoluble fraction: The first possibility was examined using cycloheximide (CHX), a protein synthesis inhibitor. Incorporation of ^{3}H-leucine into the TCA-insoluble fraction of canine tracheal smooth muscle strips was used as an index of de novo protein synthesis. A concentration of 5 mM CHX was found to result in about 95% inhibition of incorporation of the radioactive leucine.

b) Effects of CHX on isometric tension: Tissues were preincubated for 30 minutes with 5 mM CHX and later exposed to either TEA or 4-AP for one hour. Tissues were also incubated in Krebs solution either with or without 5 mM CHX for a similar period and these served as controls. Isometric tension was monitored in all these tissues. In tissues from three of six animals, preincubation with CHX resulted in a loss of mechanical activity when later treated with either TEA or 4-AP and in tissues from three others such activity was seen. All the tissue strips were fixed for thin section electron microscopy.

Structural effects of CHX:

Treatment of tissues with CHX resulted in structural damage to smooth muscle cells. The damaged cells were structurally characterized as "light" cells. "Light" cells have been observed in smooth muscles under a variety of conditions (Discussion in Chapter IV). Examination of "light" cells in the tracheal smooth muscle treated with CHX revealed damage to mitochondria, disappearance of surface caveolae and the surface membrane, suggesting the possibility of an altered metabolic state. The changes were very similar to those seen in rat myometrium by Garfield and Daniel (1976) after metabolic inhibition and mechanical injury. A greater proportion of "light" cells were encountered in tissues with no mechanical activity to TEA and 4-AP than in those where such activity was present. Thus, muscle cells were damaged by CHX to a variable extent and those extensively damaged did not show spontaneous activity.

Effects of CHX on gap junctions:

The number of gap junctions in tissues treated with CHX and either TEA or 4-AP remained higher than in the control tissues, in cases where phasic activity was evident. The number was slightly less than in tissues treated with the drugs alone. In tissues where such activity was not seen, an increase in the number of gap junctions was not observed. However, a greater number of "light" cells were seen in the latter group of tissues and the results obtained above should be interpreted in the light of the extent of such damage. Gap junctions were not observed between "light" cells and any other cells in close proximity. Thus, the failure to see any increase in some tissues could be related to extensive damage induced by CHX so that the possibility of forming gap junctions was limited by the absence of competent cells in the neighbourhood.

The continued formation of gap junctions in the absence of de novo protein synthesis in the canine tracheal smooth muscle suggests that the subunits involved in their formation are pre-formed. These pre-formed subunits could assemble into gap junctions upon arrival of specific stimuli.

viii) Freeze-fracture studies: Examination of the freeze-fracture replicas from control, TEA-, and 4-AP-treated tissues revealed extensive membrane fracture faces containing particles. The particles were seen on both the PF and the EF faces, but there were more in the PF face. The surface caveolae were observed as rows on the membrane. Assembly of particles characteristic of gap junctions were very rare in the replicas. Replicas of tissues treated with TEA and 4-AP also showed very few gap junctions. The gap junctions which were encountered were seen on cell processes. The extensive membrane fracture faces of the cells did not have gap junctions. The occurrence of gap junctions on processes as revealed by this technique thus confirms my thin section findings. The expected increase in the number of gap junctions in the TEA- and 4-AP-treated tissues could not be confirmed using this technique.

The process of fracturing results in breakage along planes of weak bonding. The process of membrane-splitting at the cell processes could be different. This could explain the difficulty in demonstrating gap junctions in this smooth muscle. Indeed the freeze-fracture technique led to the observation of fewer gap junctions per unit area than did the thin section technique. When gap junctions are in small cell processes, the technique of freeze-fracturing does not provide any additional information about gap junctions.

The various stages underlying the formation of gap junctions could not be demonstrated in this study. There was also no evidence for the presence of small gap junctions. Such structures, if present on cell processes, would be missed in thin sections. The chemical composition of the particles seen in the nonjunctional membrane is not known and there are no morphologically distinguishable features to base their distinction from the junctional subunit particles.

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